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THE EFFECTS OF LACTIC ACID BACTERIA SPECIES ON PROPERTIES OF SOUR BEER

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Tiivistelmä – Referat – Abstract

Hapanoluen aistinvaraisissa ominaisuuksissa voi tapahtua odottamattomia muutoksia hyödynnettäessä maitohappobakteerifermentaatiota. Myös toivottujen, aistinvaraisiin ominaisuuksiin vaikuttavien komponenttien tuottaminen hapanoluisiin on osoittautunut haastavaksi. Työssä tutkittiin kuuden eri maitohappobakteerin vaikutuksia hapanoluiden aistinvaraisiin ja kemiallisiin ominaisuuksiin, joita analysoitiin soveltuvin menetelmin.

Hapanoluet valmistettiin käyttäen seuraavia maitohappobakteerilajeja: *Lactobacillus plantarum*, *L. rhamnosus*, *L. brevis*, *L. buchneri*, *L. delbrueckii* sekä *L. alimentarius*, jota ei kirjallisuuskatsauksen perusteella ole aiemmin käytetty hapanoluen valmistuksessa. Pastöroimattomien oluiden valmistus kesti yhteensä noin kuusi viikkoa. Valmistuksen aikana mitattiin muutoksia solutiheydessä, pH:ssa, taitekertoimessa, orgaanisten happojen ja etanolin konsentraatiossa (käyttäen PDA/RI-menetelmää) sekä sokereiden konsentraatioissa (käyttäen HPAEC-PAD -menetelmää). Aistinvarainen arviointi suoritettiin kahdessa osiossa: Kouluttamaton raati arvioi oluita yleisen miellyttävyyden perusteella ja koulutettu raati suoritti oluille kuvailevan analyysin.

Solutiheydessä, orgaanisten happojen konsentraatioissa ja kuvailevan aistinvaraisen arvioinnin tuloksissa havaittiin tilastollisesti merkitseviä eroja. Maitohapon, etikkahapon, meripihkahapon ja etanolin konsentraatioissa havaittiin kasvua, kun taas sitruunahapon, maltoosin, glukoosin ja sakkaroosin konsentraatioissa havaittiin laskua. Aistinvaraisen arvioinnin perusteella *L. alimentarius* soveltuu hapanoluen valmistukseen. Laji myös kasvoi voimakkaasti vierteessä. *L. delbrueckiin* taas ei havaittu kasvavan lainkaan, joka johti oluen mahdolliseen pilaantumiseen tunnistamattomalla mikrobilla. *L. brevis* ja *L. buchneri* tuottivat sekä aistinvaraisessa arvioinnissa että kemiallisessa analyysissä havaittua etikkahappoa. Maitohappokonsentraation havaittiin korreloivan positiivisesti sekä oluen yleiseen miellyttävyyteen että sen vadelmaisiin, omenaisiin ja viinimäisiin ominaisuuksiin. Tämän ryhmän havaitiin taas korreloivan negatiivisesti voihappoisten, hiivaisten, härskien ja kitkerien ominaisuuksien kanssa.

Lisää tutkimuksia suositellaan fermentaatio-olosuhteiden, kuten saatavilla olevien ravinteiden ja fermentaatiolämpötilan vaikutusten arvioimiseksi hapanoluiden ominaisuuksiin.

Avainsanat – Nyckelord – Keywords
Hapanolut, maitohappobakteeri, fermentaatio, aistinvarainen arviointi
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Tiivistelmä – Referat – Abstract

In the thesis, the effects of six different lactic acid bacteria (LAB) species on properties of sour beer was studied. As the fermentation by LAB may yield unexpected results, and as the prediction of specific organoleptic compounds present in LAB-fermented sour beers has proven to be challenging, sensory and chemical properties of sour beers fermented with selected LAB was assessed. The main research question was "are there statistically significant differences between the different sensory properties of the sour beers produced with different LAB species?".

Use of *Lactobacillus plantarum*, *L. rhamnosus*, *L. brevis*, *L. buchneri*, *L. delbrueckii* and previously unreported in sour beer brewing, *L. alimentarius*, was assessed. Fermentation was carried over approximately six weeks, during which the changes in viable cell density, pH, refractive index, organic acid and ethanol (using PDA/RI) and sugar compositions (using HPAEC-PAD) were assessed. Sensory evaluation was performed in two parts: Overall preference rank test with untrained panellists and descriptive analysis with trained panellists.

Differences were found in the results of viable cell count, organic acid composition and descriptive sensory analysis. Increase in lactic, acetic and succinic acid and ethanol over the fermentation period was detected. Decrease in citric acid, maltose, glucose and sucrose was detected. Novel *L. alimentarius* yielded fastest growth rate, with encouraging results from sensory analysis. *L. delbrueckii* did not grow in the substrate media, allowing possible infection to take place. *L. brevis* and *L. buchneri* yielded acetic acid concentrations detectable in sensory and chemical analysis. Increased lactic acid concentration was associated with increased overall rank, raspberry aroma and apple and vinous flavours, with negative correlation to butyric, rancid and yeasty flavours and bitter aftertaste.

Further studies are suggested to assess the effects of fermentation temperature, O_2 availability, wort composition and buffering capacity on the results of the LAB fermentations.

Avainsanat – Nyckelord – Keywords
Sour beer, lactic acid bacteria, fermentation, sensory analysis

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
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PREFACE

This Master's thesis was done at the University of Helsinki for the Food Technology module during the academic year 2018 – 2019. The thesis was commissioned by CoolHead Brew (Plan Beer Oy, Tuusula, Finland), with the request of all acquired data being public and no information being left confidential. All aspects of the thesis were planned and carried out by the author with invaluable guidance from the members of the steering group and other personnel of University of Helsinki.

I want to express my utmost appreciation and gratitude to the members of the steering group: University Lecturer in Food Technology (Sensory Science) Antti Knaapila (PhD), who, in addition to working as the main instructor, provided outstanding guidance on sensory analysis. Professor in Food Microbiology Per Saris instructed superbly in the microbiological aspects of the thesis, and University Lecturer in Food Chemistry Velimatti Ollilainen (PhD) provided insights on the chemical analysis. Head Brewer Petteri Hänninen of CoolHead Brew acted as the professional instructor at the brewery, providing his skilful expertise on the wonderful world of beer brewing.

In addition to each member of the steering group, the would like to extend my gratitude to Senior Laboratory Technician Jutta Varis and Postdoctoral Researcher Minnamari Edelmann for their invaluable assistance in laboratory practices.

Many thanks also to Cleber Goncalves and other personnel of CoolHead Brew for the deep insights of the brewing industry, as well as to Viikki Brewing Society for arranging additional brewing equipment. Also, for generously providing the commercial control beer for the sensory evaluation, I want to extend a special thanks to James Buchanan of Thornbridge Brewery, UK and Janne Lehtinen of Brewseeker Oy, Finland. Further, I want to thank all the sensory evaluation panellists, with extended gratitude to the members of the descriptive analysis panel, who attended each session with enthusiasm and punctuality.

Finally, I cannot thank enough my family and especially my dear wife Salla, who has supported me unconditionally with her unique combination of love and science, and to whom I am ever grateful for making it possible for me to follow the paths I must follow. This is to you.

Helsinki, April 2019
Santeri Tenhovirta

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Craft brewing industry in change

As stated by Osburn et al. (2017), the modern beer world is experiencing a global craft beer boom, with the number of microbreweries growing at a tremendous pace. For example, in Finland the amount of beer producers has increased from 40 to 110 between 2013–2018 (Valvira beer producer statistics in Finland, August 2013 – August 2018). It also appears that consumers are willing to try new beers and flavours (Hong et al. 2017). For example, a significant transition from traditional bottom-fermented lager and pilsner beers towards top-fermented ales and other beer products can be noted in the beer sales in Finland (Valvira alcohol sales statistics in Finland, 2013-2017). As a result, Osburn et al. (2017) state a need for a brewery to differentiate, as the amount of competition and consumer demand have been increased. Among multiple different options of differentiation, Peyer (2017a) and Snauwert (2016) suggest sour beers for product portfolio variance, due to their unique taste and quality characteristics. De Roos and de Vuyst (2019) describe sour (acidic) beers as refreshing with fruity notes, with increasing worldwide popularity.

Sour beer is currently manufactured by utilizing the mixed fermentation of yeasts and bacteria, most commonly lactic acid bacteria (LAB) (Tonsmeire 2014). Conventional beer brewing industry regards lactic acid bacteria as spoilage bacteria, which is a justified claim when taking into consideration the beer type and consumer demands. For example, in lightly-hopped, bright and crisp lager beers LAB may produce unwanted characteristics, such as pungent acidity, buttery aromas and turbidity. However, the modern beer world is currently rediscovering the traditional sour beer styles, such as gose, Berliner weisse, lambic and Flanders red (Peyer 2017a, Tonsmeire 2014), and experimenting with novel types of microbes and fermentation techniques (Osburn et al. 2017, Nsogning Dongmo et al. 2018), yielding surprising results with commercial potential.

1.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are an order of chemoheterotrophic, gram-positive, nonsporulating and non-respiring bacteria. Von Wright and Axelsson (2012) characterize the LAB as aerotolerant cocci or rods, with lactic acid being one of their major metabolites resulting from substrate-level phosphorylation, also known as fermentation. Salvetti et al. (2012) and Pot et al. (2014) remark a growth temperature range from 2 ºC to 53 ºC, with mostly optimal

conditions between 30 ºC and 40 ºC. Further, they suggest a wide pH range of 3 – 8 in which growth occurs, with optimal pH range for growth between 5.5 – 6.2. To date, six taxonomic families of LAB are known: *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostoceae* and *Streptococcaceae,*

The genera are further branched into species, and species further to strains. Several different species and strains of LAB are used as starter cultures in a wide variety of conventionally fermented foods, such as sour bread, fermented dairy products, beverages and meat products (Serrazanetti et al. 2009; Smid and Kleerebezem 2014; Palmer 2017; Asakawa 2018).

Coda et al. (2011), Salovaara and Gänzle (2012) and Peyer (2015) mention several reasons for LAB food fermentations, emphasizing the quick acidification of the food matrix resulting in extended shelf life, as well as the resulting changes in nutritional, textural and flavour profiles, with decreased requirement for use of food additives. They explain that the acidification is due to the primary metabolite lactic acid and other organic acids, presence of which lowers the food matrix pH, and subsequently results in overall sour flavour perception of the food. Furthermore, these authors continue to maintain that with correctly performed LAB fermentations, the overall flavour and aroma compositions of the food can be positively affected via LAB metabolite bioflavours, potentially resulting in novel food products and increased enjoyability, but also to unexpected sensory responses.

Thus, Serrazanetti et al. (2009) suggests that the complex synergistical effects of external and genetic factors affecting the activities of the fermenting LAB in question in food matrices mirrors the eventual food product composition and the arising organoleptic properties of the food. Previous studies also suggest that there exists great variance of metabolomic activity even at strain level of LAB (Nsogning Dongmo 2016). For example, Bachmann et al. (2009) found significant variation in the concentration of the key aroma compound 3-methyl-butanal in gouda cheeses, ripened using 38 different *Lactococcus lactis* strains, while Kajala et al. (2018), isolating living *Lactobacillus backii* and *Pediococcus damnosus* strains from beer bottles scavenged from 170-year old shipwreck, noted that these historical versions of common LAB lacked plasmid-encoded genes, which allow their modern counterparts to survive in modern beer. Instead, the historical strains expressed genes responsible of exopolysaccharide synthesis, which are not present in their modern variations.

1.2.1 Taxonomic criterion

According to Sun et al. (2014), the genus *Lactobacillus* is the largest and most diverse group of the LAB species and therefore, is of the main interest here. Sun et al. (2014) maintain that the fermentation types occurring in different metabolic pathways are the most important taxonomic criterions for LAB species, with three distinct types of LAB metabolic pathways having been described. According to Salvetti et al. (2014), only little correlation exists between the phylogenetic relatedness and metabolic properties of different species.

The modern definition groups the genus to obligate homofermentative (OHO, group I), facultative heterofermentative (FHE, group II) and obligate heterofermentative species (OHE, group III) (von Wright and Axelsson 2012; Sun et al. 2014; Endo and Dicks 2014). These are, however, generalized outlines, and not without exceptions (von Wright and Axelsson 2012). Environmental, growth media and fermentation process conditions are known to influence on the metabolic response and on the selection of the pathway used (Serrazanetti 2009; Endo and Dicks 2014).

1.2.2 Obligate homofermentative species

OHO species ferment only hexoses nearly exclusively to lactic acid via the glycolytic Embden-Meyerhof-Parnas (EMP) pathway (Sun et al. 2014; Endo and Dicks 2014). These species are not able to ferment pentoses (Endo and Dicks 2014) and lack the phosphoketolase enzyme (Pot et al. 2014), but possess the defining enzymes fructose-1,6-diphosphate (FDP) aldolase and hexose isomerase (Jay 2000). In the pathway, glucose is phosphorylated to glucose-6-phosphate and further to FDP by ATP. The aldolase enzyme cleaves FDP to dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) (Pot and Tsakalidou, 2009).

Utilizing substrate-level phosphorylation, GAP and DHAP are converted to pyruvate, which is in turn catalysed by the enzyme lactate dehydrogenase (LDH) to lactate (Pot and Tsakalidou, 2009). In homolactic fermentation, two molecules of lactate and two molecules of ATP are generated from one molecule of glucose (Jay 2000; Endo and Dicks 2014). The species grouped here include for example *L. amylovorus, L. acidophilus, L. delbrueckii, L. helveticus* and *L. salivarius* (von Wright and Axelsson 2012).

Behr et al. (2015), studied the sugar fermentation and growth capabilities of six different LAB species in readily brewed beer. They noted that some homofermentative species can act with versatile metabolic strategies depending on available nutrients and media pH conditions, for example by counteracting on the acid secretion, thus creating buffering capacity in the media, decreasing the acid stress.

1.2.3 Obligate heterofermentative species

OHE species are capable of metabolizing hexoses, pentoses and related compounds (Endo and Dicks 2014), but instead of EMP pathway, they degrade sugars via pentose phosphate (phosphoketolase) pathway, also known as hexose monophosphate shunt (Jay 2000). In the pathway, similarly to glycolysis, glucose is phosphorylated to glucose 6-phosphate by ATP, which instead of further phosphorylation, is oxidized to ribulose 5-phosphate (von Wright and Axelsson 2012).

Ribulose 5-phosphate is further converted to xylulose 5-phosphate, which, by the defining phosphoketolase enzyme, is split into GAP and acetyl phosphate (Pot and Tsakalidou 2009). GAP is converted to lactate as in glycolysis, while acetyl phosphate is first reduced by the effect of acetyl coenzyme A to acetaldehyde, and via the oxidation of NADH to NAD^+, eventually to ethyl alcohol (von Wright and Axelsson 2012; Endo and Dicks 2014). In the presence of alternative electron acceptors, acetyl phosphate may also be converted to acetic acid (von Wright and Axelsson 2012). In this heterolactic pathway, one molecule of lactic acid, CO_2 and ethyl alcohol from one molecule of consumed glucose are in theory produced (Endo and Dicks 2014). Species included in this group are for example *L. brevis*, *L. buchneri*, *L. fermentum* and *L. reuteri* (von Wright and Axelsson 2012). Jay (2000) claims heterofermentative species being more important in producing flavour and aroma compounds, such as acetaldehyde and diacetyl, than homofermentative species.

1.2.4 Facultative heterofermentative species

FHE species possess characteristics of the both obligate metabolic pathways. Hexoses are fermented exclusively to lactic acid via EMP, and under glucose limitation, pentoses are fermented to acetic acid, ethyl alcohol and formic acid via the phosphoketolase pathway (Endo and Dicks 2014; Sun et al. 2014). However, the distinction is not restricted. For example, according to Nsogning Dongmo (2017), by effect of changes in various fermentation

conditions, the mesophilic *L. plantarum* can change the sugar metabolism from EMP glycolysis to heterolactic phosphoketolase pathway. Species in the FHE group include for example *L. plantarum*, *L. casei*, *L. curvatus* and *L. sakei* (von Wright and Axelsson 2012).

1.2.5 Metabolite and flavour compound variance

The formation of flavour is described as accumulation of volatile and non-volatile compounds, which, in LAB fermentation, are mostly organic acids, complemented by amino acids, alcohols, aldehydes, ketones, oligopeptides and carbonyl compounds in varying quantities and concentrations (Smid and Kleerebezem 2014; Peyer 2015). As stated by Peyer (2015) and Asakawa et al. (2018), the physiological abilities of different LAB species and strains to use different types of nutrients from the media during fermentation results in great variance of metabolite production. As a result, Peyer (2015) estimates that predicting a presence of specific organoleptically active compounds or bioflavours in a sour beer may be challenging, however, different LAB species can give characteristic and contrasted properties to the beer.

1.3 Sour beer

In conventional beer brewing industry, the presence of LAB is generally associated with beer spoilage (Kunze 1999; Behr et al. 2015; Kim et al. 2016; Asakawa et al. 2018). In contrast, LAB is regarded as a crucial element in the formation of beer flavour and aroma of some beer styles. Tonsmeire (2014), Spitaels (2014, 2015a,b) and Peyer (2017b) describe several such styles, such as the Belgian lambic, gueuze, Flanders red, and the German berliner weisse, all of which have long histories in the European beer culture.

Sour beer brewing methods differ from conventional brewing only by introducing LAB and/or other wort-souring microbes at some point during the manufacturing process (Tonsmeire 2014; Osburn et al. 2017; Peyer 2017b). Tonsmeire (2014), Osburn et al. (2017) and Peyer (2017b) describe several different methods of sour beer production, which can be divided to two distinct methods: Barrel aging, taking years to complete, and sour worting (divided to kettle souring and tank souring methods), which results in finalized beer in approximately one month. In a study by Peyer (2015), LAB fermentation was found to introduce complexity into the overall flavour of the beer, with each strain inoculation exhibiting different kinds of flavour profiles.

1.4 Objectives

The objective of the thesis was to study how different LAB species affected sensory characteristic, as well as the organic acid and sugar concentrations of sour beer. Thus, the aims of the experimental research were the following:

- To produce seven different beers, six of which inoculated with different LAB species, with one nonacidified control beer inoculated with *Saccharomyces cerevisiae* only,
- to study the performances of the fermentations,
- to study the sugar and organic acid changes in each produced beer,
- to study the sensory properties of each produced beer,
- and to study the possible relations between the chemical and sensory properties.

The study was commissioned by CoolHead Brew (Plan Beer Oy, Tuusula, Finland, later: the brewery). As of beginning of 2019, the brewery has manufactured approximately 150 batches of beer, estimated half of which have been sour beers flavoured with various fruits and berries. The company has experimented on and currently produces sour beers with limited amount of LAB species, mainly *L. rhamnosus*, but lack detailed, in-depth knowledge regarding how different species affect the sensory properties of final beer.

As the company has transitioned from their previous wort souring method of kettle souring to tank souring, a variation of tank souring method was used in this study for the acidification of the beer. Since tank souring method permits LAB activity during beer maturation, sensory properties of sour beers were of primary interest. The brewery was also interested in the possibly unexpected results arising from the use of novel LAB species which they had not yet used in brewing.

The objectives of the thesis were to study how the different LAB species affected the fermentation and sensory properties of sour beer, and to study how the resulting variances in carbohydrate and organic acid compositions affected the sensory properties of the produced beers. Also, the feasibility of using *L. alimentarius* in sour beer manufacture was explored, a species with, to the best of knowledge, no prior documented deliberate use in sour beer manufacture has been previously attempted on.

The sole independent variable was the LAB species. Dependent outcome variables were organic acid concentrations, fermentable carbohydrate concentrations, pH levels and the results of the sensory analysis. Also, the acidification performance of each bacteria, measured in hours to reach static and terminal pH levels, was observed.

By choosing the LAB species as the sole independent variable and keeping the media composition and fermentation conditions otherwise identical, it was therefore expected that only the actions of each separate LAB species had effect on the dependent outcome variables of the beer samples.

Primary research question was "*are there statistically significant differences between the different sensory properties of the sour beers produced with different LAB species?*", and secondary "*how the actions of different LAB species affect sour beer organic acid and fermentable carbohydrate compositions during fermentation and maturation, and do the occurred changes have statistically significant relation to the results of sensory evaluations?*".

2 EXPERIMENTAL RESEARCH

2.1 Materials and methods

2.1.1 Test materials

The grist composition resulted from replicating and down-scaling an industrial sour beer recipe grain bill used by the brewery in their commercial sour beers. In the recipe, mash thickness, also known as liquor to grist ratio, was 2.83 l of mashing water per 1 kg of malt used. Resulting from the maximum equipment volume of 35 litres per batch and following the down-scaled grist composition, presented in Table 1, mash water quantity resulted 19.8 litres per batch.

Table 1. Grist composition per one batch of manufactured beer.

Ingredient	Manufacturer	kg
Pilsner malt	Viking Malt Oy	2.67
Wheat malt	Viking Malt Oy	2.67
Wheat flakes	Raisio Oyj	0.92
Oat Flakes	Raisio Oyj	0.76

The grain bill raw materials were donated by the brewery. The used raw materials were pilsner malt (Viking Pilsner Malt crushed, batch no. 8938, packing date 31.8.2018, Viking Malt Oy, Lahti, Finland), wheat malt (Viking Wheat Malt crushed, batch no. 8008, packing

date 29.4.2018, Viking Malt Oy, Lahti, Finland), wheat flakes (Nalle täysjyvävehnähiutale, best before 9.2.2019, Raisio Oyj, Raisio, Finland) and oat flakes (Elovena täysjyväkaurahiutale, best before 5.7.2019, Raisio Oyj, Raisio, Finland). Water used for all process stages was tap water.

Majority of the bacteria inoculum was purchased from University of Helsinki Microbial Domain Biological Resource Centre Culture Collection HAMBI. Only the *L. rhamnosus* was a commercially available freeze-dried inoculum (Lyofast LRB, Sacco srl, Cadorago, Italy) and was selected due to it currently being used as the main acidification bacteria at the brewery. The species selected from the HAMBI collection were selected based on the availability, prior reported usage history and respective metabolic pathway used. To accompany the OHO *L. rhamnosus*, one additional OHO species, two OHE species and two FHE species were to be selected. One requirement was to study at least one species with no prior reported use in sour beer, albeit in other sectors of the food industry.

From the HAMBI collection, *L. alimentarius* was selected due to no previous reported use in sour beer manufacture. Previous studies report the use of *L. alimentarius* in the manufacture of tarhana, a Middle Eastern fermented grain-based soup (Özdemir et al. 2018) and in traditional Chinese fermented pork meat, nanx wudl (Hu et al. 2017). In literature there are conflicting descriptions of the metabolic pathway of *L. alimentarius*: Özdemir et al. (2018) detected carboxylic acid in fermentations inoculated with *L. alimentarius*, suggesting heterofermentative behaviour. Sun et al. (2014), with de Vuyst et al. (1994) and Pot et al. (2014) referencing Reuter (1983a), claim the species is FHE, while Reuter himself (1983a and 1983b), Lyhs et al. (2000) and Jay (2000) maintain the species as homofermentative. This division is further accented in the determination of a taxonomic *Lactobacillus alimentarius* group (Salvetti et al. 2012; Pot et al. 2014; Sun et al. 2014), comprising of 11 *Lactobacillus* species (Sun et al. (2014) claiming 12), members of which express both OHO and FHE pathways. The metabolic pathway of the *L. alimentarius* HAMBI 411 strain remained thus unresolved.

Other HAMBI species were FHE *L. plantarum*, OHE *L. brevis*, OHE *L. buchneri* and OHO *L. delbrueckii*, all of which having prior reported use in sour beer manufacture. LAB species used in this thesis, with their corresponding metabolic pathways, lot and HAMBI codes and knowledge of prior reported use are presented in Table 2. The HAMBI species were delivered from the collection on MRS agar plates.

Table 2. Lactic acid bacteria species used, with respective lot or HAMBI catalogue numbers. FHE: Facultative heterofermentative. OHE: Obligate heterofermentative. OHO: Obligate homofermentative.

LAB species	Metabolic pathway	Lot / HAMBI catalogue number	Previous reported use in brewing
L. plantarum	FHE	HAMBI: 382	At brewery[2], in literature
L. brevis	OHE	HAMBI: 76	In literature
L. buchneri	OHE	HAMBI: 69	In literature
L. rhamnosus	OHO	Lot: C160136A	At brewery[2], in literature
L. delbrueckii	OHO	HAMBI: 1427	In literature
L. alimentarius	–[1]	HAMBI: 411	None

1)Unresolved.
2)CoolHead Brew, Tuusula, Finland.

Reference food products and reagent details for the descriptive sensory analysis are described in Table 3. Full ASBC reference attributes list is presented in appendix 1.

Table 3. Food and reagent reference samples for corresponding ASBC reference codes.

ASBC Reference code	Attribute[1]	Food or reagent reference	Serving portion and/or concentration[2]	Make, origin or manufacturer	Lot# / Batch# / Best before
0112	Vinous (F)	White wine, alc. vol 5.5%	40 ml	Hardy's Stamp of Australia	Manuf. 2017
0141	Citrus (F)	Lemon juice, bottled	20 ml	Xtra Sitruunatäysmehu	BB 031219
0142	Apple (F)	Apple, fresh	One slice	Royal Gala, Italy	Purch. 110219
0147	Raspberry (A)	Raspberry, fresh	Three raspberries	BerryWorld, Morocco	531013
0160	Floral (A)	-[3]	-	-	-
0320	Malty (A)	Pilsner malt, ground	20 ml	Viking Malt Oy, Finland	Batch 8938
0614	Butyric (F)	Butyric acid	40 ml, 20 mg l^{-1}	Sigma-Aldrich, USA	STBH8638
0630	Rancid (F)	Propionic acid	40 ml, 90 mg l^{-1}	Sigma-Aldrich, USA	STBH0545
0740	Yeasty (F)	Baker's yeast	40 ml, 4000 mg l^{-1}	Suomen Hiiva Oy, Finland	BB 280219
0910	Acetic (F)	Acetic acid	40 ml, 200 mg l^{-1}	VWR, USA	15G290514
0920	Sour (F)	Lactic acid	40 ml, 1200 mg l^{-1}	Merck, USA	K11448466
1200	Bitter (AT)	Caffeine	40 ml, 1500 mg l^{-1}	Sigma-Aldrich, USA	BCBJ6076V
1340	Astringent (AT)	Alum	40 ml, 1000 mg l^{-1}	Yliopiston Apteekki, Finland	YL9902838

1) Attribute type abbreviations: (F) Flavour, (A) Aroma, (AT) Aftertaste.
2) Reagent solutions 614-1340 diluted in tap water, served at ambient temperature.
3) Floral attribute reference was not determined, subjective "floral" experiences of panellists were encouraged instead.

The yeast inoculum was commercial *S. cerevisiae* strain US-05, (SafAle US-05, freeze-dried, Fermentis, S.I. Lesaffre, Marcq-en-Barœul, France, lot 37140/005, best before 01/2021), selected due to wide reported use (Peyer et al. 2017; Liu and Quek 2016; Canonico et al. 2016). According to the manufacturer, the yeast cell count was > 6×10^9 viable cells g^{-1} (SafAle US-05 Technical datasheet, revision Nov. 2016).

Prior to each use, all fermentation vessel surfaces and other brewing equipment in contact with wort and beer were treated with Star San HB (Five Star Chemicals & Supply, Inc., Commerce City, CO, USA. Exp.lot 28.4.2022, 042816, later: Star San), diluted with distilled water to 0.3 ml l^{-1} concentration, in accordance to the MSS of the manufacturer. Food-grade CO_2 for beer bottling was supplied by Suomen Kuivajää Oy, Nastola, Finland. Priming sucrose for bottle conditioning used was Dansukker Siro erikoishieno sokeri (Suomen sokeri Oy, Kantvik, Finland, batch no. 1832876121). Commercial control beer for sensory analysis was Thornbridge Tart Bakewell Sour, best before 13th July 2019, donated generously by Thornbridge Brewery (Rakewell, UK) and Brewseeker Oy (Helsinki, Finland). The reagents for the internal standard solutions for the chemical analyses are presented in Table 4.

Table 4. Reagents used for the standard solutions in the PDA/RI and HPAEC-PAD analyses.

Component	Reagent# / product#	Manufacturer	Purity	Lot# / Batch#	Concentration(1 mg ml^{-1}
Sugars					
Maltose	5912	Merck, USA	99 %	6374901	1.046(2 / 0.104(3
Glucose	1.08337.0250	Merck, USA	99 %	2122497	0.998(2 / 0.106(3
Fructose	1.04007.0250	Merck, USA	99 %	2610	1.009(2 / 0.112(3
Sucrose	S-7903	Sigma, USA	≥ 99.5%	44H06512	0.100(3
Acids					
Lactic acid		Merck, USA	90 %	K11448466	0.999(2
Acetic acid	20103.330	VWR, USA	99-100%	15G290514	0.998(2
Propionic acid	W292400-1KG-K	Sigma, USA	≥ 99.5%	STBH0545	1.000(2
Butyric acid	W222100-1KG-K	Sigma, USA	≥ 99%	STBH8638	0.998(2
Citric acid	C1909500G	Sigma, USA	≥ 99.0%	120M0186V	1.086(2
Succinic acid	S7501-500G	Sigma, USA	99 %	-	0.257(2
Alcohol					
Ethyl alcohol	Etax Aa	Altia Oyj, Finland	≥ 99.5%	18385	1.000(2

1) Diluted in ultrapure Milli-Q water (Merck Millipore, USA) and filtered with 0.45 µm syringe filter.
2) Concentration for the PDA/RI detection
3) Concentration for the HPAEC-PAD detection

2.1.2 Brewing process

A flowchart of the complete brewing process is depicted in Figure 1. Three batches of beer base wort were prepared simultaneously with three identical Bulldog Brewer brewing equipment (Hambleton Bard Ltd., UK), with each batch volume being approximately 30 litres.

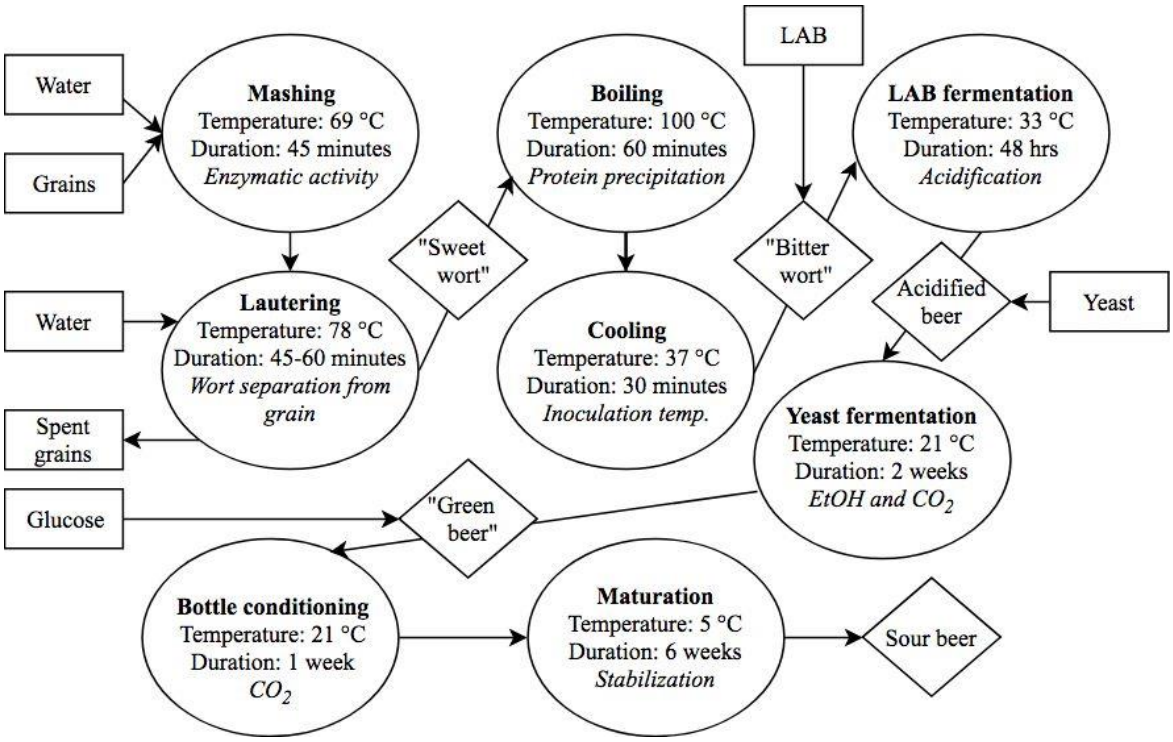

Figure 1. Flowchart of sour beer manufacturing process for this thesis.

In mashing, the ground and mixed grains, now called grist, were combined in the Bulldog Brewer's malt baskets with mashing water and mixed thoroughly. Mashing temperatures were set to 69 °C with Bulldog Brewer temperature controls. The mashes rested in this temperature for a duration of 45 minutes. After the mashing period, first wort runnings were circulated with the Bulldog Brewer pump systems for one minute on top of the malt beds for clarification.

Sweet wort was collected in the lautering process. Malt baskets containing the spent grain were lifted on top of the boiling vessel, allowing remaining sweet wort to sieve through the bed, collecting to boiling vessel below. Once the flow was depleted, additional sparging water of 79 °C was ran through the malt bed, resulting in approximately 29 litres of sweet wort collected per brewing unit. Lautering lasted from 45 to 60 minutes, depending on brewing equipment unit. Once collected, the worts were boiled with the brewing units for 60 minutes in a vigorous, rolling boil.

Worts were cooled with a heat exchanger to approximately 37 °C, measured from several locations in the wort using a digital thermometer (Multi-Thermometer, Brouwland, Belgium). Final yield was approximately 84 litres of bitter wort in total. To achieve uniform wort composition, worts were transferred to a 50-litre collection vessel in two parts, first combining half of the worts of all three brews. From the collection vessel, the mixed wort was divided to six LAB fermentation vessels. Once the collection vessel was emptied, the procedure was repeated. Each separate LAB fermentation vessel yielded approximately 8.5 litres in total. The wort remaining at the collection vessel, approximately 33 litres, was fermented as the nonacidified control beer. After cooling the wort further with a heat exchanger to approximately 21 °C, the wort was inoculated with *S. cerevisiae* US-05 dry yeast by sprinkling the yeast on the wort surface, with yeast quantity of 0.7 g l^{-1}, resulting in approximately 4×10^6 viable cells ml l^{-1} of wort, mixing the yeast in by agitating the vessel slightly. The vessel was sealed with an airlock. No deliberate acidification was thus performed on the control beer.

The six separated worts were inoculated aseptically with one LAB species each. For each of the five HAMBI species, 5 ml of the wort was pipetted to each MRS agar plate containing the bacterial colonies, allowing the wort to hydrate the colonies. The colonies were then carefully mixed to the wort with a glass stirring rod, avoiding to mixing in the MRS growth media. For the freeze-dried commercial *L. rhamnosus* bacteria, a starter inoculum was prepared. 100 ml of wort was measured into a sterile bottle, and the available bacteria (approximately 0.5 g measured aseptically from a commercial, previously unopened *L. rhamnosus* pouch stored at approximately -12 ºC) was mixed in using a glass rod. The starter inoculum was kept at 38 °C for approximately 30 minutes to allow bacterial hydration and initial growth phase to take place. Each LAB fermentation vessel was then inoculated by pouring contents on top of each wort, mixing the inoculum into the wort by agitating the vessel slightly. Each vessel was sealed with an airlock. The ambient air contained in the fermentation vessels was left to remain within, and not purged with CO_2 as opposed to industrial brewing settings, in which fermenting beer would be protected from oxidation.

The nonacidified control beer fermentation vessel was placed at ambient temperature of approximately 21 °C. The ambient temperature was read from the room thermometer. The LAB fermentation vessels were placed to a temperature-controlled room at approximately 38 °C.

After allowing 41 hours of acidification, each LAB fermentation vessel was placed at ambient temperature of approximately 21 °C. Once the temperature of the fermentation vessels had decreased to ambient temperature, approximately 65 hours after the LAB inoculation, yeast was inoculated to each of the fermentation vessel with yeast quantity of 0.7 g l^{-1}, resulting in viable yeast cell density of approximately 4×10^6 cells ml^{-1} of beer. 50 ml of distilled water was boiled, measured to a Falcon tube and allowed to cool down to ambient temperature of approximately 21 °C. Freeze-dried yeast was mixed aseptically into the water and allowed to hydrate for 10 minutes. The inoculums were poured into the fermentation vessels aseptically and mixed by agitating the vessels carefully in a rotational fashion.

2.1.3 Monitoring the acidification, fermentation and viable bacterial count

Acidification, fermentation and bacterial growth were monitored during the whole fermentation process. To monitor the acidification, pH levels were measured with Mettler Toledo SevenCompact S220-Basic pH meter (Mettler Toledo, Inc., Columbus, OH, USA). To monitor fermentation, total soluble solids was measured in °Brix (later: °Bx) with Brouwland ATC refractometer (Brouwland bvba, Beverlo, Belgium). Samples of 4 ml were taken aseptically from each fermentation vessel by using a pipette, using the same sample quantity for all monitoring requirements. The airlock of each LAB fermentation vessel was removed for sample taking, treating the airlock and surrounding area with Star San before and after of removal. The samples were measured as taken from the fermentation vessels, with no pre-treatments performed. For the first week of the acidification and fermentation processes, the measurements were taken approximately between 24 hours, after which the measurements were taken approximately between 48 hours. At sensory evaluation (960 hours), terminal pH was measured with Milwaukee pH55 -pH meter (Milwaukee Electronics Kft., Szeged, Hungary).

To monitor bacterial growth, plate cultivation was used to determine viable bacterial count. On the brewing day, immediately after bacterial inoculation, samples were cultivated to determine initial bacterial inoculation rates. 100 µl of each of the inoculated fermentations were diluted with 900 µl of MRS broth. A serial dilution was performed until a 10^{-5} dilution of the original fermentation sample was reached. Of the 10^{-5}, 10^{-4}, and 10^{-3} dilutions, three 10 µl droplets of each dilution were pipetted aseptically on MRS agar plate, arriving at viable count suspensions of 10^7, 10^6 and 10^5 CFU ml l^{-1}, respectively. The plates were placed in the same temperature-controlled room with the LAB fermentation vessels, in a temperature

of approximately 38 °C. After 48 hours of incubation the visible colonies on the 10^7 CFU ml l^{-1} suspension droplet areas were counted and thus CFU ml l^{-1} concentration in each fermentation vessel was determined. The viable count was determined in identical fashion from each of the LAB fermentations also after the acidification period (before the yeast inoculation), and after the primary fermentation before bottling.

2.1.4 Bottling, bottle conditioning and maturation

When a stationary phase was attained in each fermentation vessel by following the depletion of fermentable solids, the beer was bottled. A 20-litre cornelius keg was used as a temporary bottling vessel. Prior to use and between bottling each fermentation, the internal keg surfaces were sanitized first by heating with nearly boiling water and treating afterwards with Star San. CO_2 was pushed inside the cornelius keg, replacing ambient air.

Since no viable options was available to carbonate the beer by CO_2 injection to exact levels, as is the brewing industry method of beer carbonation, CO_2 had to be generated by secondary, anaerobic fermentation in each bottle. Since each of the fermentations had reached a stationary phase, sucrose was added to initiate the secondary fermentation. The brewery sour beer recipe targeted 2.4 – 2.6 litres of eventual CO_2 per 1 litre of beer. Equation (1), designed for homebrewers and mixing imperial and metric units (Hall 1995), was used to calculate the amount of required sucrose to reach desired CO_2 volume in beer.

$$M_{sucrose} = 15.195\, V_{beer}\, (V_{CO_2} - 3.0378 + 5.0062 \cdot 10^{-2}T - 2.6555 \cdot 10^{-4}T^2) \quad (1)$$

In the equation, $M_{sucrose}$ = the mass of sucrose required (g), V_{beer} = volume of the beer (gallons), V_{CO2} = targeted volume of CO_2 per litres of beer (L) and T = temperature of the beer in degrees Fahrenheit. Thus, with each beer, 6.25 g of sucrose per 1 litre of beer was dissolved in 50 ml of boiled distilled water and added aseptically into the keg. The beer was then siphoned from the fermentation vessel to the keg, leaving settled yeast and other solids inside the fermentation vessel.

Beer added with secondary fermentation sucrose was bottled from the keg with Blichmann beer gun (Blichmann Engineering, LLC, Lafayette, IN, USA). The bottles were generic brown glass long-neck beer bottles of 0.33 litre volume. Star San was sprayed inside each bottle prior to bottling, and allowed to drip dry, storing upside-down. At bottling, beer gun was used first to remove the ambient air inside the bottle by pushing CO_2 inside the bottle,

and immediately to fill the bottle with beer, thus minimizing the O_2 exposure of beer during bottle-conditioning and maturation. Bottles were sealed with generic crown caps.

Bottles were then stored at ambient temperature of approximately 21 °C for one week to allow the secondary, anaerobic fermentation to occur, generating CO_2 in the beer. The bottles were then moved to a temperature-controlled room at approximately 5 °C for maturation and stabilization for 6 weeks until the sensory evaluation and chemical analysis.

2.1.5 Performance of sensory analyses

The sensory analyses were performed in accordance to the ASBC Methods of Analysis, Sensory Analyses 1-13 (later: Method number 1-13). Sensory analysis data was collected both computer-assisted and manually. For computer-assisted data collection and sample presentation order randomization, Fizz version 2.51 (Biosystemes, Couternon, France, later: Fizz) was used.

The sensory analysis was divided to two parts. The first part, performed with an untrained panel (n=20) in one session, consisted of an overall preference ranking test, performed in accordance to Method number 11: Ranking test. In the test, all eight beer samples were served simultaneously to each panellist once in randomized order. The samples, approximately 40 ml per beer, were served in see-through plastic cups under ambient lighting conditions, at approximate serving temperature of 5 °C. The panellists were asked to smell and taste each beer and rank the samples in their subjective overall preference order, ranging from least favourable to most favourable. The samples were not swallowed. Using Fizz, each panellist gave each beer sample a unique Likert scale value between 1 (least preferred) and 8 (most preferred). Screen captures of the Fizz user interface screens are presented in appendix 2.

The second part, a descriptive analysis performed in accordance to Method number 10: Descriptive analysis, was divided to four sessions, each performed on different days during the same week. The first two sessions were training sessions for the actual evaluation, which was performed in the two latter sessions. The descriptive analysis evaluation panel consisted of eight panellists, who were trained in the first two training sessions.

In the first training session, panellist trainees (n=8) were served seven samples of beer in randomized order with unique codes dissimilar to the overall preference test. The sample

quantity was approximately 40 ml per sample, served under similar conditions to the overall preference test. Due to small available amount of *L. delbrueckii*, this sample was omitted from the first training session. The trainees were provided with a handout of ASBC standard attribute list for odour, taste, mouthfeel, warming and after flavour (Method number 12: Flavor terminology and reference standards, appendix 1). The trainees were then instructed to smell and taste each beer sample in order, focusing on one beer at a time. When detecting flavours, odours or mouthfeel attributes in the beer samples, the trainees were asked to mark the corresponding term on the attribute list by using the beer codes and corresponding attribute type (flavour, aroma or aftertaste attribute). The number of attribute instances were then counted, and 11 most prominently occurring attributes were selected. Thus, the vocabulary for the second training and later evaluation sessions was collected.

In the second training session, reference samples based on the collected attributes were served to the panellist trainees under similar conditions to the first training session. Each of the reference samples were served simultaneously on trays in see-through plastic cups, each marked with corresponding ASBC reference code. The trainees were then instructed to familiarize with the reference samples by smelling and tasting. Discussion among the panel members about the reference samples was encouraged. After the reference sample familiarization, the references were removed and replaced with six beer samples, served in see-through plastic cups, approximately 40 ml quantity per beer, and each uniquely coded dissimilar to the first training session samples. The omitted beer samples in the second training session, due to available beer quantity, were the commercial control beer and *L. rhamnosus*. The trainees were then instructed to attempt at detecting the referenced attributes in the sample beers and marking the detected attributes on to a new ASBC attribute handout (appendix 1), similarly to the first training session. Further, if novel attributes were still to be detected, these were also instructed to be marked. Discussion regarding the beer samples and attributes among the trainees was again encouraged. Once each of the trainees had sampled the beers, the sample flavours and aromas were discussed openly. By the results of these discussion sessions, the addition of floral aroma (ASBC reference 0160) and yeast flavour (0740) to the selected attribute list were prominently encouraged and were thus included. Abbreviated list of the collected attributes is presented in Table 5 with food and reagent reference samples, presented in the order of the evaluation. The complete attribute list with corresponding ASBC reference codes along with reference sample food and reagent details is presented in Table 3. These selected 13 attributes were transferred to the Fizz software.

Table 5. Abbreviated attribute and reference sample list in the order of evaluation presentation. Detailed list is presented in Table 3.

Attribute	Food or reagent reference
Floral (A)	-
Malty (A)	Pilsner malt, ground
Raspberry (A)	Raspberry, fresh
Citrus (F)	Lemon juice, bottled
Apple (F)	Apple, fresh
Vinous (F)	White wine, alc. vol 5.5%
Sour (F)	Lactic acid
Acetic (F)	Acetic acid
Yeasty (F)	Baker's yeast
Butyric (F)	Butyric acid
Rancid (F)	Propionic acid
Bitter (AT)	Caffeine
Astringent (AT)	Alum

In the sensory evaluation sessions of descriptive analysis, the trained panellists (n=8) were served all eight beer samples simultaneously, in randomized order, 40 ml per beer, in see-through plastic cups covered with a plastic lid, under red-tinted lighting to mask the appearance of the beers. Each beer sample was presented uniquely coded with randomized three-digit codes. The reference samples were available during the evaluation. The sensory evaluation was performed in duplicate on successive days, with unique sample codes and randomized serving order for each day, recording the evaluations of each panellist from both days.

Based on the distinct division of aroma, flavour and aftertaste attributes (Table 5), the attributes were divided in four groups: Aroma (floral, malty and raspberry aromas), flavour 1 (citrus, apple, vinous and sour flavour), flavour 2 (acetic, yeast, butyric and rancid flavour) and aftertaste (bitter and astringent aftertaste). The panellists were asked to assess each group completely before moving to the next group. First, the intensities of aroma attributes of each sample were evaluated. The evaluation was performed by sniffing one sample at a time and assessing the intensity of the specific attribute. By using water as the reference of lack of the attribute in the sample and the corresponding reference sample as a theoretical maximum value for the attribute in question, the panellist placed a marker on to the provided visual analogue scale, representing their evaluation of the intensity of the attribute in the sample. After evaluating the aroma attributes of each samples, the panellist evaluated the flavour and aftertaste attributes. While performed otherwise similar, the panellist was instead asked to taste the sample and spit the sample out after tasting. Thus, the intensities of each attribute

in each sample was evaluated, similar attributes evaluated in groups. User interface screens for the descriptive sensory analysis are presented in appendix 3.

2.1.6 Performance of chemical analyses

Samples were collected from four different moments in the fermentation process, at 0, 65, 378 and 960 hours. Samples from wort (0 hours), from each fermenting vessel after acidification period (prior to yeast addition, 65 hours), from each vessel after the yeast fermentation period (378 hours), and finally from the matured bottles (960 hours) were collected and analysed to determine sugar, organic acid and alcohol concentrations of each bacteria during these steps in brewing. The measured sugar concentrations were those of maltose, glucose, fructose and saccharose. The measured organic acid concentrations were those of lactic, acetic, citric, succinic, butyric and propionic acid. For alcohol, the ethyl alcohol concentration in the samples was measured.

Each sample was frozen prior to analysis to prevent microbial actions to occur during storage. Upon analysis, the samples were thawed in refrigerator temperatures overnight. The thawed samples were treated with ultrasonic agitation with Branson 5510R-MT Ultrasonic Cleaner (Branson Ultrasonics, Danbury, CT, USA) to remove excess CO_2 from solution. Using ultrapure Milli-Q water purified with Millipak Express 40 0.22 µm membrane filter (Merck Millipore, Burlington, MA, USA), the samples were diluted to 1:5 of the original for PDA/RI analysis and to 1:25 of the original for HPAEC-PAD analysis. The diluted samples were transferred to 1 ml autosampler vials, filtering the samples through GHP Acrodisc 0.45 µm glass fibre syringe filters.

For the analysis of maltose, glucose, fructose and sucrose, HPAEC-PAD (High-Performance Anion-Exchange Chromatography and Pulsed Amperiometric Detection) method was performed, utilizing Waters 2707 autosampler (Waters Corp., Milford, MA, USA), Waters 515 HPLC pump (Waters Corp., Milford, MA, USA), SSI pulse dampener model LP-21 (Scientific Systems Inc., State College, PA, USA), CarboPac PA-1 Guard columns (4 x 50 mm) (Dionex Corporation, Sunnyvale, CA, USA), CarboPac PA-1 anion exchange columns (4 x 250 mm) (Dionex Corporation, Sunnyvale, CA, USA), Waters 2465 pulsed amperiometric detector (PAD) (Waters Corp., Milford, MA, USA) and Empower2 software for data collection (Waters Corp., Milford, MA, USA). The temperature of the column was 30 ºC for a runtime of 60 minutes per sample, while the used eluent was a gradient of 200 mM NaOH

in concert with Milli-Q water. The eluent gradient is included in appendix 4. The samples, with injection volumes of 2 µl, were analysed in single.

For the analysis of organic acids and ethyl alcohol, a PDA/RI (Photodiode Array / Refractive Index) method was used, utilizing Waters e2695 Separations Module (Waters Corp., Milford, MA, USA), Hewlett-Packard HP 1047A RI Detector (Hewlett-Packard Inc., Palo Alto, CA, USA), Waters 996 Photodiode Array Detector (Waters Corp., Milford, MA, USA), Hi-Plex H HPLC column (300 x 6.5 mm) (Agilent Technologies, Inc., Santa Clara, CA, USA), and Empower2 software for data collection (Waters Corp., Milford, MA, USA). The temperature of the column was 40 ºC with a runtime of 60 minutes per sample. The used eluent was 10 mM H_2SO_4. The samples, with injection volume of 40 µl, were analysed in duplicate.

For both methods, internal standard solutions were constructed. Containing the reagents presented in Table 4, the chromatogram peak locations for each of the standard solution components were determined and thus used to assess the retention times for each of the detected components. From each of the methods, chromatograms of each analysed samples were acquired. By comparing the component retention times from internal standards and by integrating the peak areas of all recognized peaks, concentrations for each of the detected components were calculated. An example chromatogram from PDA/RI is presented in appendix 5.

2.1.7 Statistical analyses

For all statistical analyses described here, IBM SPSS Statistics version 25 (IBM Corporation, Armonk, NY, USA, later: SPSS) was used. One-way ANOVA with Tukey's post-hoc test was used to determine the statistical significance of the following: Viable cell count from the MRS agar cultivations, results of the sensory evaluations and organic acid concentrations in readily fermented beers. In addition, Friedman test was used to determine the statistical significance of the results of the overall preference ranking test of the sensory analysis. To perform the principal component analysis (PCA), Origin 2018b (OriginLab Corp., Northampton, MA, USA; later: Origin) was used. To generate figures unless otherwise stated, Microsoft Excel version 1902 (Microsoft Corp., Redmond, WA, USA) was used.

2.2 Results

2.2.1 Fermentation tracking

The beer pH levels decreased rapidly during the first 48 hours from the initial wort pH of 5.89 (Figure 2). After 41 hours of acidification and transferring the LAB beers from 38 ºC to 21 ºC, the decrease rate of pH was slowed down, but continued to decline further in each of the beers. After reaching their lowest pH levels 130 to 200 hours after the LAB inoculation, ranging from pH 3.02 (*L. plantarum*) to pH 3.76 (*L. delbrueckii*), a notable increase in pH levels started to occur. During bottling, 378 hours after the LAB inoculation, pH levels ranged from pH 3.18 (*L. rhamnosus*) to pH 4.01 (*L. delbrueckii*).

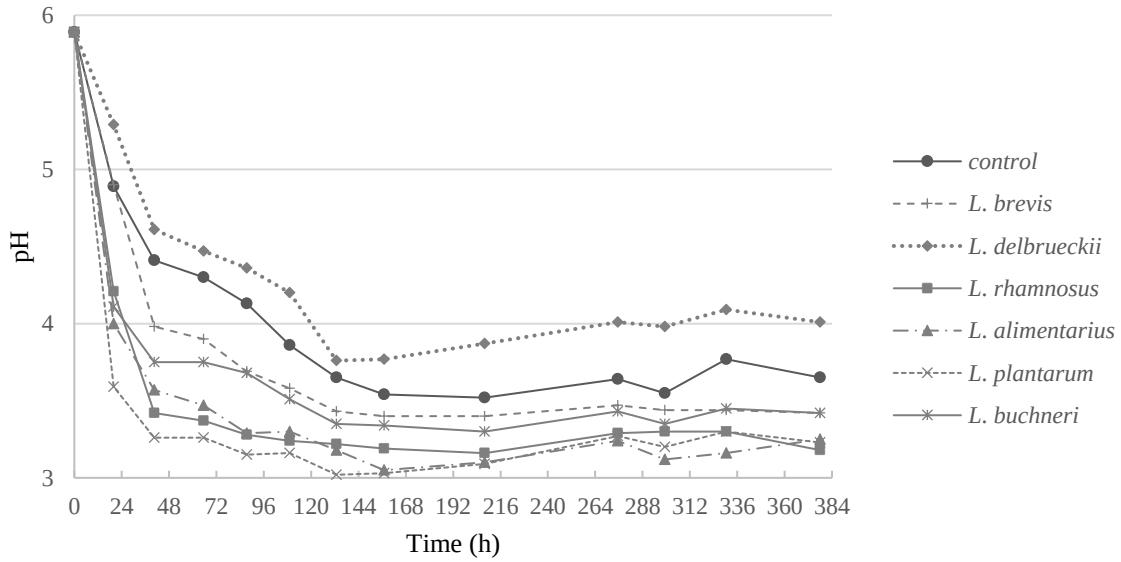

Figure 2. pH values as measured during fermentation. Y-axis begins at pH 3.0.

A slowdown of the pH decrease was observed between the 41-hour and 65-hour time frame, when the LAB fermentation vessels were transferred from 38 ºC to 21 ºC. This occurred with control beer as well, which had remained at ambient 21 ºC. Quite notably, *L. delbrueckii* had the highest pH values during fermentation, exceeding even the nonacidified control beer, suggesting low acidifying activity. Furthermore, a rapid decrease of pH in nonacidified control beer should be noted.

L. plantarum reached lowest pH levels fastest, with the commercial *L. rhamnosus* showing slightly slower activity. The previously untested *L. alimentarius* appeared to acidify the beer at approximately similar rate with *L. rhamnosus*, reaching only slightly lower pH values at lowest (pH 3.03 after 157 hours).

The eventual pH levels, as measured at 378 hours (bottling) and 960 hours (sensory analysis), indicating changes while in storage, are presented in Table 6. Most change during maturation occurred in *L. rhamnosus* with increase from pH 3.18 to 3.40. With *L. delbrueckii* and *L. brevis* the pH appeared to decrease, while with *L. plantarum*, *L. alimentarius* and *L. buchneri*, there appeared to be moderate increase.

Table 6. pH changes in each beer during maturation.
N=1 for each measurement.

Sample	pH	
	378 h	960 h
L. plantarum	3.23	3.30
L. alimentarius	3.25	3.30
L. rhamnosus	3.18	3.40
L. brevis	3.42	3.40
L. buchneri	3.42	3.50
L. delbrueckii	4.01	4.00

The °Bx of wort prior to microbial inoculations was measured to be 13 °Bx. As can be observed from Figure 3, solid matter of control beer started to decrease rapidly 20 hours after the inoculation. This indicates a 20-hour lag phase in control beer, followed by an exponential phase of the yeast fermentation. After approximately 65 hours including the lag phase, the fermentation pace seemed to decrease. The exponential phase was followed by a slower decrease from approximately 7 °Bx to approximately 6 °Bx during a period of 92 hours. Afterwards, the value remained relatively stationary for approximately 9 days, until bottling.

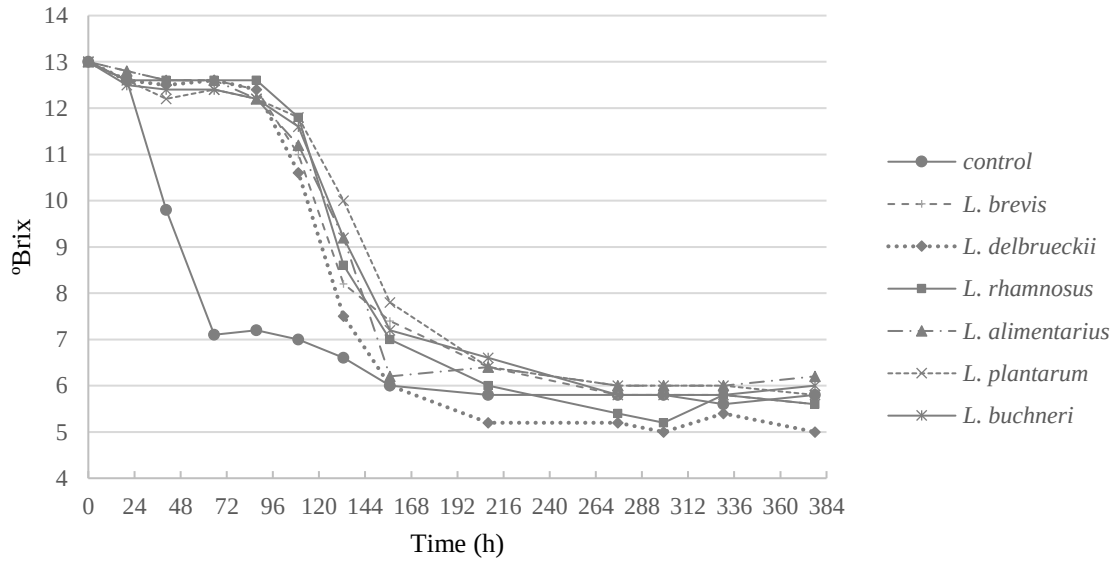

Figure 3. °Brix values as measured during fermentation. For control beer, yeast inoculation is at 0 hours. For LAB fermentations, bacterial inoculation is at 0 hours and yeast inoculation at 65 hours. Y-axis begins at 4 °Bx.

For each of the LAB fermentations, a rapid decrease in °Bx values started approximately 22 hours after yeast inoculation, indicating a lag phase followed by an exponential phase of yeast fermentation. Despite the strongly acidified media of sour beers (pH 5.89 during yeast inoculation in the control beer, compared to pH 3.7 ± 0.4 during yeast inoculation in the sour beers), the performance of yeast fermentation seemed to be relatively similar in both environments, each phase taking only longer to occur in the acidified media. For the sour beers, the °Bx decreased from the approximate initial 12.5 °Bx to average of 6.9 ± 0.7 °Bx during a period of 92 hours including the lag phase, lasting thus approximately 27 hours longer in the acidified beers than in the control beer. Furthermore, the decline of °Bx values continued slowly, establishing relatively stable values ranging from 157 to 234 hours after the yeast inoculation (control beer and *L. rhamnosus,* respectively). The eventual average °Bx value for all acidified beers at bottling was 5.7 ± 0.4 °Bx.

L. delbrueckii reached the °Bx value of control beer approximately 92 hours after yeast inoculation, continuing to ferment even further. While control beer remained at average 5.8 ± 0.1 °Bx for nine days after reaching a stable level, *L. delbrueckii*-inoculated beer continued to ferment to a stable level of 5.2 ± 0.2 °Bx, where it remained for seven days.

An important aspect when considering the °Bx values is the refractive index of water (n=1.333 at λ=589 nm, Hecht 2002, Table 4.1) compared to that of ethyl alcohol (n=1.36 at λ=589 nm, Hecht 2002, Table 4.1). When measuring actively fermenting solutions containing increasing amounts of ethyl alcohol, the current °Bx values are always given in relation to the refractive index of the current ethyl alcohol concentration in water solution. Thus, subsequent °Bx measurements of actively fermenting solutions with increasing concentrations of ethyl alcohol are not comparable to preceding measurements of the same solution, but in fact give increasingly higher values. This results that the actual solids concentration of the solution is in fact lower than measured. Considering this, the measured °Bx values in Figure 3 are thus progressively non-comparable and lower than depicted, however, correct for the original unfermented wort.

The plate cultivation of the sour beer samples revealed not only the approximate bacterial inoculation rates for each of the beers (Table 7, 0 hours), but also the bacterial growth in each beer (65 – 378 hours).

Table 7. CFU count for each species in 10^7 CFU ml l^{-1} at inoculation (0 hours), after acidification before yeast inoculation (65 hours) and at bottling (378 hours). Measurements were done in triplicate.

Species	0 h	65 h	378 h
L. brevis	$1 \pm 0^{(a}$	$4 \pm 2^{(d}$	$26 \pm 4^{(f}$
L. delbrueckii	0 ± 0	0 ± 0	0 ± 0
L. rhamnosus	$20 \pm 1^{(c, ***}$	$1 \pm 1^{(2, d}$	$49 \pm 9^{(g, *}$
L. alimentarius	$1 \pm 0^{(a}$	$56 \pm 4^{(e, ***}$	$78 \pm 6^{(h, **}$
L. plantarum	$3 \pm 1^{(1, ab}$	$8 \pm 3^{(d}$	$27 \pm 0^{(f}$
L. buchneri	$6 \pm 2^{(b}$	$8 \pm 1^{(d}$	$12 \pm 1^{(f}$
Control	- (3	- (3	$1 \pm 1^{(2}$

1) CFU count was determined from 100 µl plate cultivation.
2) In 10^5 CFU ml l^{-1}. No other growth or colonies were found on the plate.
3) Not plated.
a-h) Statistically homogenous subsets marked with corresponding letters as resulted from Tukey's. Statistical comparisons are for each column respectively.
*) The mean difference is significant at $p \leq 0.05$
**) The mean difference is significant at $p \leq 0.01$.
***) The mean difference is significant at $p \leq 0.001$.

L. delbrueckii did not produce any visible colonies on any of the plates, and thus probably had low viable bacterial count ($\leq 1 \times 10^5$ CFU ml l^{-1}) in the sour beer media. This suspicion was supported by the minimal acidification of the *L. delbrueckii* -inoculated beer (Figure 2), as well as the extended decrease rate of fermentable solids (Figure 3), suggesting low acidic inhibition of yeast growth.

L. brevis, *L. alimentarius* and *L. plantarum* were inoculated at statistically homogenous rate (Table 7, subset a). *L. plantarum* and *L. buchneri* formed also a homogenous subset of inoculation rate (Table 7, subset b). *L. rhamnosus* was inoculated at a significantly higher rate compared to other species ($p \leq 0.001$, Table 7, subset c), unsurprisingly due to much greater volume of bacterial inoculum (visually approximated 0.5 g of freeze-dried *L. rhamnosus* compared to limited number of colonies of plate-cultivated HAMBI species).

65 hours after bacterial inoculation, before yeast inoculation, *L. alimentarius* had increased bacterial count significantly compared to other species ($5.6 \pm 4 \times 10^8$ CFU ml l^{-1}, $p \leq 0.001$, Table 7, subset e). Nearly every other species formed a homogenous subset in CFU count (Table 7, subset d), excluding *L. delbrueckii* with no visible colonies on any plate. At 65 hours after inoculation, the cell count of *L. rhamnosus* was determined to be $1 \pm 1 \times 10^5$ CFU ml l^{-1} with no other colonies visible on the plate. At such a low CFU count, errors in sample-taking or plating procedures were suspected, notably when the CFU counts of *L. rhamnosus* remained much higher before, at inoculation, and later, before bottling.

378 hours after bacterial inoculation, before bottling, CFU count of *L. alimentarius* had remained significantly higher compared to other species ($78 \pm 6 \times 10^7$ CFU ml l^{-1}, $p \leq 0.01$, Table 7, subset h). Further, the CFU count of also *L. rhamnosus* had increased significantly ($4.9 \pm 9 \times 10^8$ CFU ml l^{-1}, $p \leq 0.05$, Table 7, subset g). The remaining species formed a homogenous subset (Table 7, subset f), excluding again *L. delbrueckii* with no visible colonies. The results indicate that the LAB species continued to grow regardless of competitive environment after *S. cerevisiae* inoculation at 65 hours. The nonacidified control beer was plated only at 378 hours after inoculation, producing $1 \pm 1 \times 10^5$ CFU ml l^{-1}.

In all plating instances (0, 65 and 378 hours), the CFU counts of the OHE *L. brevis* and the FHE *L. plantarum* belonged to same homogenous subsets (a, d, f), with *L. plantarum* forming another group of subsets with the OHE *L. buchneri* (b, d, f). Other species had distinctly unique growth behaviour, noting foremost the lack of visible colonies in any of the *L. delbrueckii* plates, and the sudden colony count decrease in the 65-hour sample of *L. rhamnosus.* Quite interestingly, the previously untested *L. alimentarius* performed surprisingly well, having formed more colonies already at 65 hours after inoculation ($5.6 \pm 4 \times 10^8$ CFU ml l^{-1}, $p \leq 0.001$, n=3) than any other species at 378 hours after the inoculation.

2.2.2 Sensory properties

By summing the Likert scale values given for each beer in the overall preference ranking test, rank sums and rank mean values (n=20) were calculated (Table 8). One-way ANOVA and Friedman test were performed for the Likert scale results with SPSS. The overall preference test did not reveal statistically significant results in the given rank values.

Table 8. The results of the overall preference ranking test (n=20).

Sample	Rank sum	Rank mean
Non-acidified control (CtrlNonA)	103	5.15 ± 2.25
Commercial control (CtrlComm)	96	4.80 ± 2.14
L. plantarum	94	4.70 ± 2.12
L. brevis	93	4.65 ± 2.15
L. rhamnosus	90	4.50 ± 2.01
L. buchneri	88	4.40 ± 2.52
L. alimentarius	81	4.05 ± 2.16
L. delbrueckii	75	3.75 ± 2.97

Revealed by the rank sum and rank mean, the non-acidified control beer was evaluated as being the most preferred beer, with rank sum of 103 and rank mean of 5.15 ± 2.25 (n=20).

The commercial control was succeeded by the non-acidified control beer. Of the LAB-acidified beers, FHE *L. plantarum* was ranked the highest with rank sum of 94 and rank mean of 4.70 ± 2.12. *L. delbrueckii* was ranked the lowest with rank sum of 75 and rank mean of 3.75 ± 2.97.

For the results of the aroma, flavour and aftertaste attributes evaluated by the trained panel in the descriptive analysis, statistically significant differences between the beers were found in evaluated intensities of floral aroma, citrus flavour, yeasty flavour ($p \leq 0.001$), in vinous flavour, sour flavour and malty aroma ($p \leq 0.01$), and in raspberry aroma ($p \leq 0.05$). No statistically significant results were found in the evaluated intensities of apple flavour, acetic flavour, butyric flavour, rancid flavour, astringent aftertaste or bitter aftertaste. The results of the descriptive analysis are presented in Figure 4, and including homogenous subsets, in appendix 6.

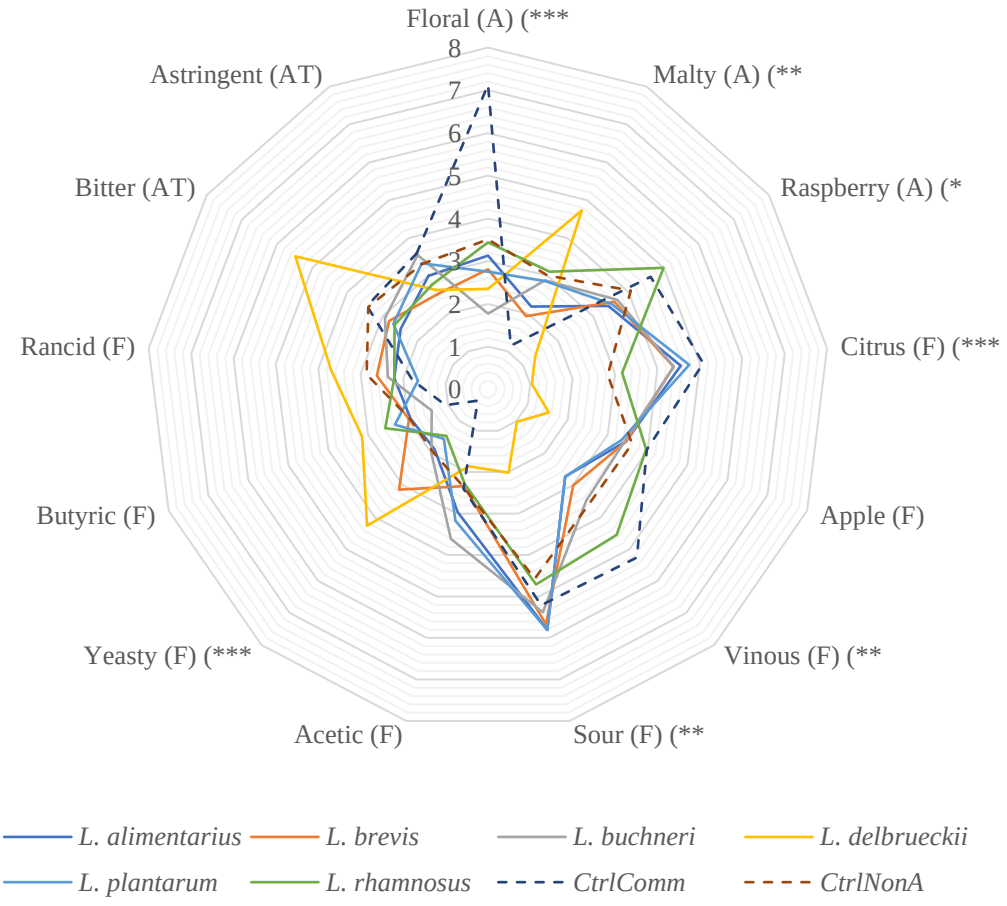

Figure 4. The results of the descriptive analysis. N=16 for each attribute and beer (8 panellists, evaluations in duplicate). Each scale was ranked from 0 to 10 on a visual analogue scale. The attributes mean differences are statistically significant at *) $p \leq 0.05$; **) $p \leq 0.01$; ***) $p \leq 0.001$ significance levels (1-way ANOVA). Results with homogenous subsets as revealed by the Tukey's test are presented in tabular form in appendix 6.

As depicted in the radar chart (Figure 4) and homogenous subsets presented in appendix 6, the floral aroma, citrus flavour and yeasty flavour attributes of the beers were evaluated most significantly different ($p \leq 0.001$ for each attribute). For floral aroma and citrus flavour, the commercial control beer was given the highest scores of 7.13 ± 2.75 and 5.08 ± 3.24, respectively, forming a single homogenous subset in the intensity of floral aroma. No significant differences were found between the floral aroma intensities of the beers brewed for this thesis, but in the citrus flavour intensity evaluation, *L. plantarum*, *L. alimentarius*, *L. brevis* and *L. buchneri* were evaluated as forming a homogenous subset with the commercial control. The citrus flavour of *L. delbrueckii* was evaluated lowest, forming a single homogenous subset. Furthermore, *L. delbrueckii* formed a single homogenous subset also in the yeasty flavour, having the highest value of all the samples of 4.27 ± 3.15.

The second most significantly different results ($p \leq 0.01$) were in the vinous flavour, sour flavour and malty aroma. The commercial control was evaluated as having the highest value of vinous flavour, forming a homogenous subset with *L. rhamnosus* (5.26 ± 2.90 and 4.55 ± 3.40, respectively). A middle group belonging in both high and low subsets was formed in descending order by nonacidified control, *L. buchneri*, *L. brevis*, *L. plantarum* and *L. alimentarius*. The least vinous was *L. delbrueckii*, forming a single homogenous subset with value of 1.02 ± 1.62.

For the sour flavour, the highest-evaluated *L. plantarum* and *L. alimentarius* were evaluated quite similarly (5.81 ± 2.82 and 5.80 ± 2.58, respectively). In the same subset, the subsequent beers were in descending order *L. brevis*, *L. buchneri* and commercial control. Forming a middle group belonging in both subsets were *L. rhamnosus* and non-acidified control beer, leaving *L. delbrueckii* in a single subset with lowest value of 2.01 ± 2.64.

On the other hand, *L. delbrueckii* received the highest value of 4.73 ± 3.04 in the malty aroma evaluation, forming yet again a single homogenous subset. Commercial control beer and *L. brevis* were in the lowest subset group with values of 1.15 ± 1.93 and 1.93 ± 2.05, respectively, leaving every other sample in a middle subset group.

The differences in raspberry aroma were evaluated statistically significant at level of $p \leq 0.05$. With highest value of 5.00 ± 3.01, *L. rhamnosus* formed a subset with the commercial control beer. *L. delbrueckii* formed a single homogenous subset with lowest value of 1.34 ± 1.21, leaving every other beer in a middle-value subset.

No statistically significant differences were found in the other attributes (astringent aftertaste, apple flavour, acetic flavour, butyric flavour, rancid flavour or bitter aftertaste). However, as can be observed in Figure 4 and from the statistical analyses, *L. delbrueckii* was evaluated repeatedly either highest (yeasty flavour, butyric flavour, rancid flavour, bitter flavour and malty flavour) or lowest (raspberry aroma, citrus flavour, apple flavour, vinous flavour, sour flavour and acetic flavour), standing out as a separate entity in the radar chart.

2.2.3 Sugar content

Fermentable sugar concentrations for each beer after 0, 65, 378 and 960 hours of fermentation are presented in Figures 5 – 7 for maltose, glucose and sucrose, respectively. N=1 sample for each measurement. As each sugar concentrations were measured from singular samples, no statistical analysis was meaningful to perform.

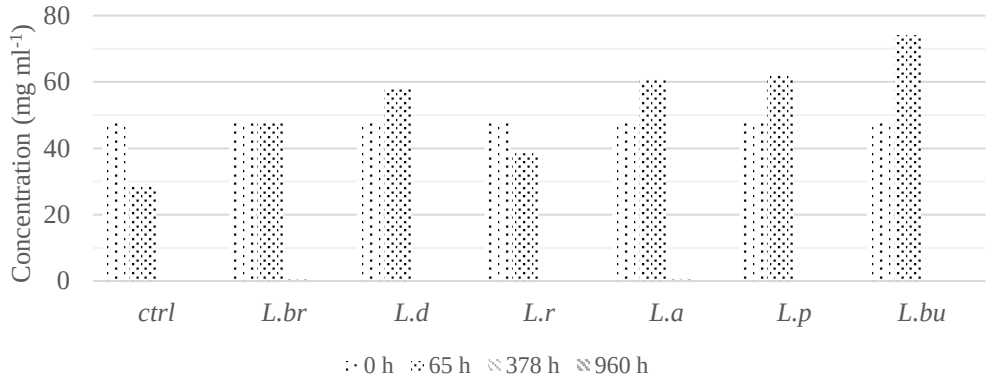

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Figure 5. Changes in concentrations of maltose. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

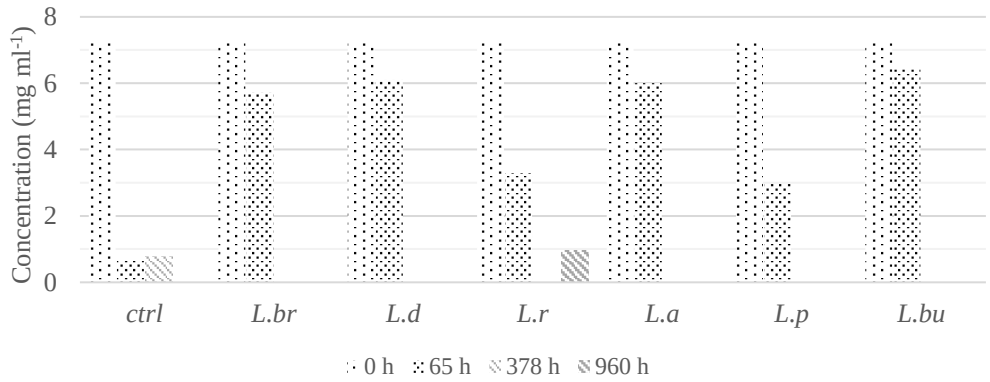

Figure 6. Changes in concentrations of glucose. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

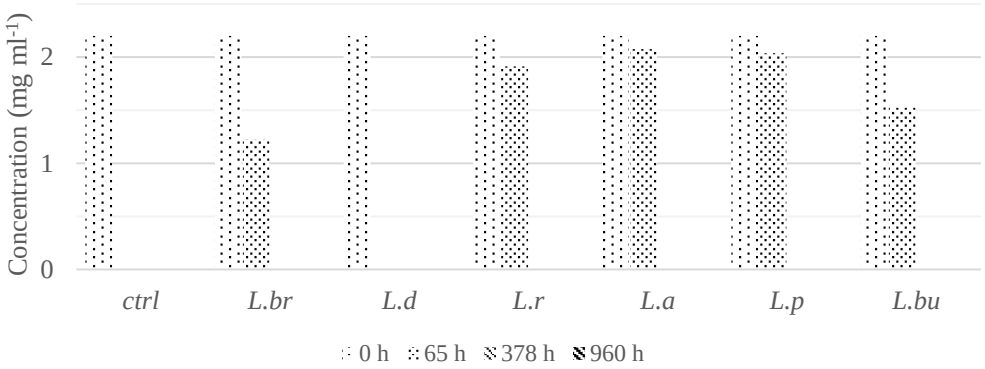

Figure 7. Changes in concentrations of sucrose. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

Topmost it is *worth* noting that besides glucose in *L. rhamnosus*, none of the samples appeared to contain the measured sugars during the sensory evaluation (960 h), indicating full metabolism of all measured sugars. This is further accented by the lack of any type of "sweet" attribute arising in descriptive sensory analysis. However, as the concentrations of maltotriose, dextrin, maltotetraose or other residual sugars was not measured, the effects of carbohydrates to the sensory attributes of the beers is difficult to assess.

The initial wort sample (0 h in each figure) contained most maltose (approximately 47.90 mg ml^{-1}), followed by glucose (approximately 7.20 mg ml^{-1}) and least sucrose (approximately 2.20 mg ml^{-1}). These values were the starting points of the measured changes. In most cases the measured quantities were decreased as expected, but in the case of *L. delbrueckii*, *L. alimentarius*, *L. plantarum* and *L. buchneri*, the quantities of maltose were increased as the LAB fermentation was progressed from 0 to 65 hours (Figure 5). This is explained possibly by error in the measured concentration of maltose in the initial wort, as apparent increase is not supported by any literature reference. However, by the time of bottling (378 hours), most maltose appeared to have been consumed after the introduction of yeast at 65 hours in LAB beers. Only *L. alimentarius* and *L. brevis* were measured to contain trace amounts of maltose at 378 hours (0.62 and 0.72 mg ml^{-1}, respectively). At sensory evaluation (960 h), none of the samples was measured to contain maltose.

In addition to the yeast consumption of glucose in the nonacidified control beer between 0 and 65 hours, decreases from the initial 7.20 mg ml^{-1} to approximately 3.30 and 3.00 mg ml^{-1} of glucose occurred with *L. rhamnosus* and *L. plantarum*, respectively (Figure 6). For other LAB-fermented beers, the measured decrease of glucose between 0 and 65 hours of fermentation was from the initial 7.20 mg ml^{-1} to 6.05 ± 0.29 mg ml^{-1} (n=4). By the time of sensory

evaluation (960 h), only *L. rhamnosus* was measured to contain glucose at concentration of approximately 0.95 mg ml^{-1}.

The initial sucrose level in each beer was approximately 2.20 mg ml^{-1} (Figure 7). In both control beer and *L. delbrueckii*, sucrose had been consumed by 65 hours into the fermentation. The OHE species *L. brevis* and *L. buchneri* were measured to consume sucrose most of the LAB species (decrease from initial concentration to 1.38 ± 0.21 mg ml^{-1} (n=2) at 65 hours) as compared to the other species (2.01 ± 0.08 mg ml^{-1} (n=4) at 65 hours). After the initial acidification period of 65 hours, and as *S. cerevisiae* had been introduced to each fermentation vessel, the concentrations of sucrose were seemingly depleted between 65 – 378 hours of fermentation in each beer.

2.2.4 Organic acid content

Organic acid concentrations for each beer after 0, 65, 378 and 960 hours of fermentation is presented in Figures 8 – 11, for lactic acid, acetic acid, succinic acid and citric acid, respectively. N=2 measurements of separate samples for each data point. One-way ANOVA analysis with Tukey's test for the concentration means of each organic acid at 960 hours revealed statistically significant differences. The differences are marked on each figure by designated homogenous subsets. The results of 960 h measurements are presented in tabular form in appendix 7.

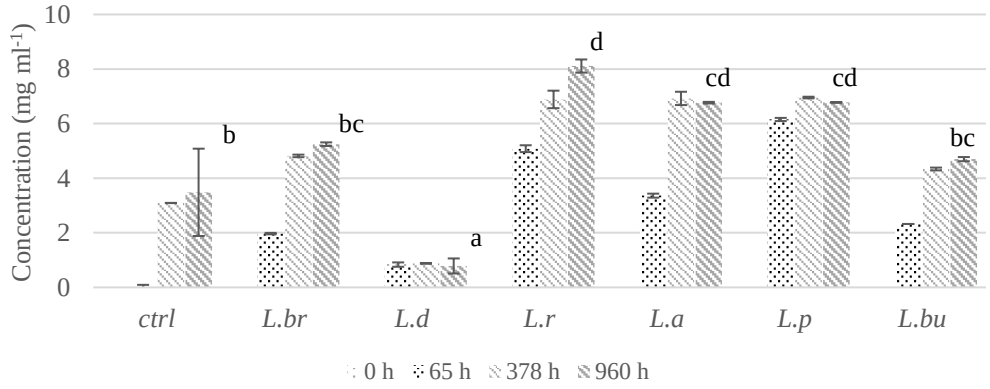

Figure 8. Changes in concentrations of lactic acid. Homogenous subsets a – d ($p \leq 0.001$, performed with Tukey's test), n=2 measurements from separate samples. Error bars signify standard deviation. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

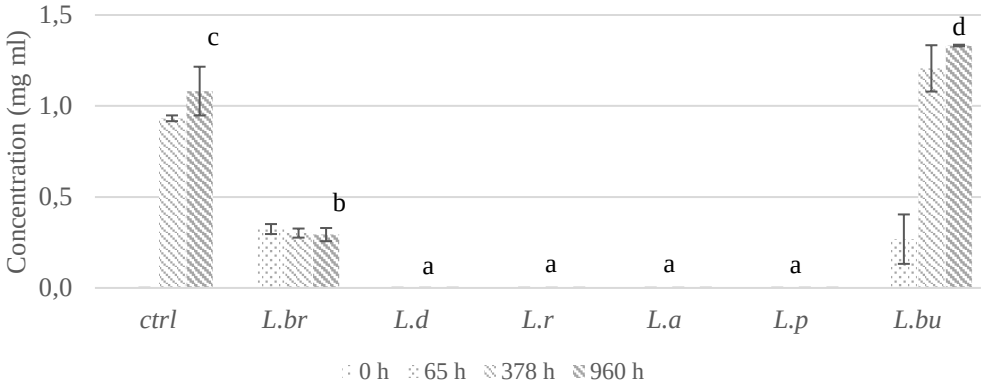

Figure 9. Changes in concentrations of acetic acid. Homogenous subsets a – c ($p \leq 0.001$ performed with Tukey's test), n=2 measurements from separate samples. Error bars signify standard deviation. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

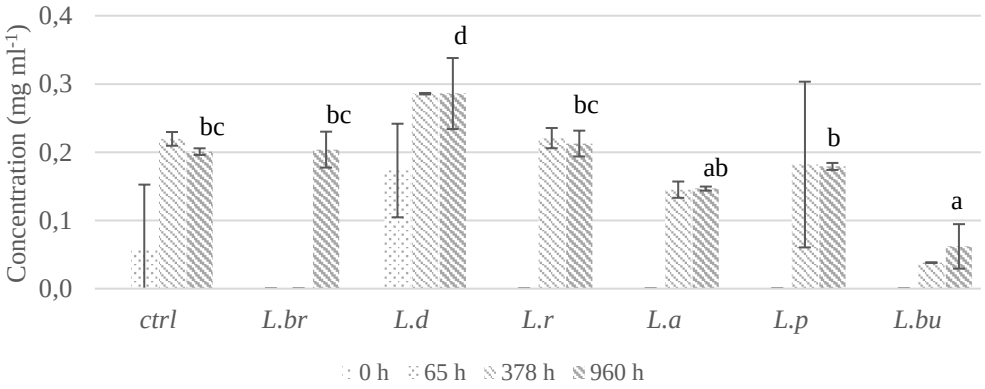

Figure 10. Changes in concentrations of succinic acid. Homogenous subsets a – d ($p \leq 0.01$, performed with Tukey's test), n=2 measurements from separate samples. Error bars signify standard deviation. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

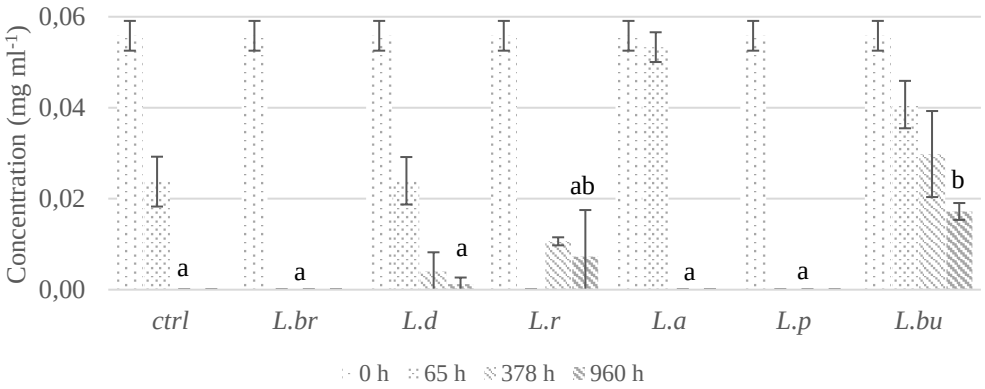

Figure 11. Changes in concentrations of citric acid. Homogenous subsets a – b ($p \leq 0.05$, performed with Tukey's test), n=2 measurements from separate samples. Error bars signify standard deviation. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

The terminal concentrations of lactic acid in the beers at 960 hours was measured to be divided to 6 homogenous subsets (a – d, Figure 8), with some beers vacating several subsets. *L. delbrueckii* was the only species in the subset (a) with lowest lactic acid concentration of the measured beers (0.78 ± 0.00 mg ml^{-1}). Further, it should be noted that *L. delbrueckii* was the only beer in which no increase was detected: Once the lactic acid concentration had reached the terminal level as measured at 65 hours into fermentation, no further changes were detected. This was contrasted by the actions of every other beer, including the nonacidified control beer, belonging in the subset (b), with terminal lactic acid concentration of 3.48 ± 1.60 mg ml^{-1} at 960 hours. Both OHE species, *L. brevis* and *L. buchneri* formed the subset (bc), with average lactic acid concentration of 4.97 ± 0.08 mg ml^{-1} (n=4). Further, the species *L. alimentarius* and *L. plantarum* formed the subset (cd) with average lactic acid concentration of 6.77 ± 0.02 mg ml^{-1} (n=4). Lastly, the commercially used *L. rhamnosus* was measured to produce the most lactic acid of the compared species, forming a single homogenous subset (d), with terminal lactic acid concentration of 8.11 ± 0.24 mg ml^{-1} (n=2). Further with *L. rhamnosus*, the lactic acid production seemed to continue to increase from 378 h to 960 h, during which every other species had reached their respective terminal concentrations. In every produced sample, the lactic acid concentrations exceeded the reported taste threshold in beer (approximately 0.45 mg ml^{-1}, Siebert 1999).

Of the LAB, only the OHE species *L. buchneri* and *L. brevis* produced notable amounts of acetic acid, with notable concentrations detected in the nonacidified control beer as well (Figure 9), with each beer forming a single homogenous subset (d, b and c, respectively). Four remaining species formed a homogenous subset (a) with no detectable concentrations of acetic acid in the samples. The taste threshold of acetic acid (0.20 mg ml^{-1} in beer, Siebert 1999) was exceeded more clearly in *L. buchneri* (1.33 ± 0.00 mg ml^{-1}) than in *L. brevis* (0.29 ± 0.39 mg ml^{-1}). Despite this was not significantly detected in the sensory analysis, an indication of the sensory detection of the produced acetic acid was clearly shown in the case of *L. buchneri* by having the highest evaluation of acetic flavour. Quite surprisingly, however, *L. plantarum*, *L. alimentarius* were given higher values compared to *L. brevis* in the evaluation of acetic flavour, despite the lack of detected acetic acid in these samples.

Succinic acid was detected in each of the beers, forming several homogenous subsets (Figure 10). Most notable is the detection of succinic acid in most cases only after yeast introduction. An exception to the LAB beers is the succinic acid concentrations in *L. delbrueckii*:

In this, the acid was detected already at 65 hours in concentrations relating to the terminal concentrations of every other beer, resulting in significantly higher terminal concentration than in any other beer, forming a single homogenous subset (d) with concentration of 0.29 ± 0.05 mg ml^{-1}, which is also above the reported succinic acid detection threshold of 0.20 mg ml^{-1} in beer (Engan 1973). Succinic acid was detected also in the nonacidified control beer at 65 hours. In other LAB beers, succinic acid was detected only after yeast introduction at 378 hours, excluding *L. brevis*, in which the first detection occurred only at 960 hours. The lowest succinic acid levels were for *L. buchneri*, which formed a single homogenous subset (a) with terminal concentration of 0.06 ± 0.05 mg ml^{-1}. In most cases, the succinic acid concentrations remained at or below the detection threshold.

In contrast to every other measured organic acid, instead of increasing, the quantities of citric acid decreased in each sample (Figure 11). The decrease was most rapid in *L. brevis* and *L. plantarum,* both in which the citric acid concentration decreased from the initial 0.06 ± 0.05 mg ml^{-1} to no detected concentration between 378 – 960 hours. *L. buchneri*, *L. rhamnosus* and *L. delbrueckii* contained statistically significant amounts ($p \leq 0.05$) of citric acid at concentrations of 0.017 ± 0.00 mg ml^{-1} (subset (b)), 0.007 ± 0.01 mg ml^{-1} (subset (ab)) and 0.001 ± 0.00 mg ml^{-1} (subset (a)). Each of these, however, remained below the reported detection threshold of 0.35 mg ml^{-1} of citric acid in beer (Engan 1973).

2.2.5 Ethyl alcohol content

Ethyl alcohol concentrations for each beer after 0, 65, 378 and 960 hours of fermentation is presented in Figure 12. Both nonacidified control beer and *L. delbrueckii* formed a homogenous subset (b) with significantly higher ethyl alcohol concentrations than in the five other samples forming a second homogenous subset (a) (59.55 ± 0.84 mg ml^{-1} (n=2) and 53.45 ± 1.36 mg ml^{-1} (n=4) respectively. $P \leq 0.001$.)

An interesting detail is the lack of notable decrease in °Bx in any of the sour beers prior to yeast inoculation (Figure 3). This indicates that most of the LAB species, excluding the OHE *L. brevis* and *L. buchneri*, did not produce detectable concentrations of ethyl alcohol during the acidification, as was confirmed by the chemical analysis (Figure 12). Ethyl alcohol concentrations for *L. brevis* and *L. buchneri* at 65 h, before yeast inoculation, were 0.28 ± 0.39 mg ml^{-1} and 0.87 ± 0.10 mg ml^{-1}, respectively. The production of ethyl alcohol,

and as well as the acetic acid production in only these two species, confirms their heterofermentative nature and encourages the suitability of these species for manufacture of sour beers with detectable acetic acid concentrations.

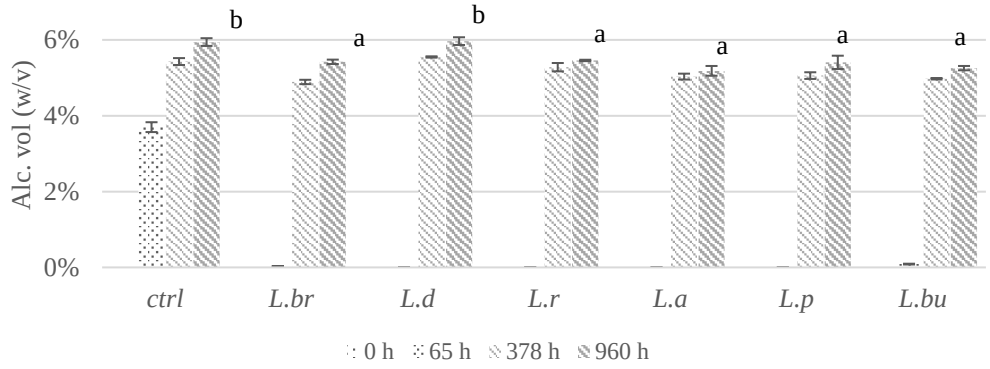

Figure 12. Changes in concentrations of ethyl alcohol between 0-960 h. Homogenous subsets a – b ($p \leq 0.001$, performed with Tukey's test), n=2 measurements from separate samples. Error bars signify standard deviation. Ctrl = Non-acidified control, *L.br = L. brevis; L.d = L. delbrueckii, L.r = L. rhamnosus; L.a = L. alimentarius; L.p = L. plantarum; L.bu = L. buchneri.*

2.2.6 Principal component analysis

By statistically combining the results of the sensory analysis and chemical analysis for each beer, a principal component analysis (PCA) was performed with Origin. According to the PCA, first four primary components, having eigenvalue of ≥ 1, explain 93.57 % of the variation within the data. However, for the sake of clarity in the results interpretation, only first two components are discussed here, taking in consideration that principal component (PC) 1 explains 56.45 % of the variation within the data, while PC 2 explains 18.69 % of the variation, resulting in a combined explanation of 75.14 % of the variation within the data. The results of the four component coefficients are presented in appendix 8. The two most significant components, PC 1 and PC 2, are bi-plotted in Figure 13.

By combining all relevant variables, PCA revealed some interesting aspects of the results. Taking in consideration the not statistically significant results of the ranking test, the rank sum vector (Rank sum, green in Figure 13) is regardless grouped with raspberry aroma (Raspberry, blue), vinous flavour (Vinous, blue) and apple flavour (Apple, blue) attributes and the concentration of lactic acid (LacticA, red). This indicates positive correlation between those variables: As the concentration of lactic acid in the sample was increased, coincidentally increased were the rank sum and the evaluated values of raspberry aroma, apple flavour and vinous flavour. Grouped nearby this positive correlation group is *L. rhamnosus*,

which indeed had the significantly highest lactic acid concentration (8.10 ± 1.60 mg ml^{-1}, $p \leq 0.001$), highest raspberry aroma evaluation and highest apple and vinous flavour evaluations of the brewed samples. Nearby the rank sum evaluation vector are *L. brevis*, *L. alimentarius* and *L. plantarum*, along with non-acidified control beer, as well as the sour and citrus flavour evaluations. Thus, as also these flavour evaluations are rated higher, also the rank sum is increased. Each of these attributes have thus somewhat positive effect on the outcome of the overall impression of the beer, especially in the case of *L. rhamnosus*, *L. brevis*, *L. alimentarius* and *L. plantarum.*

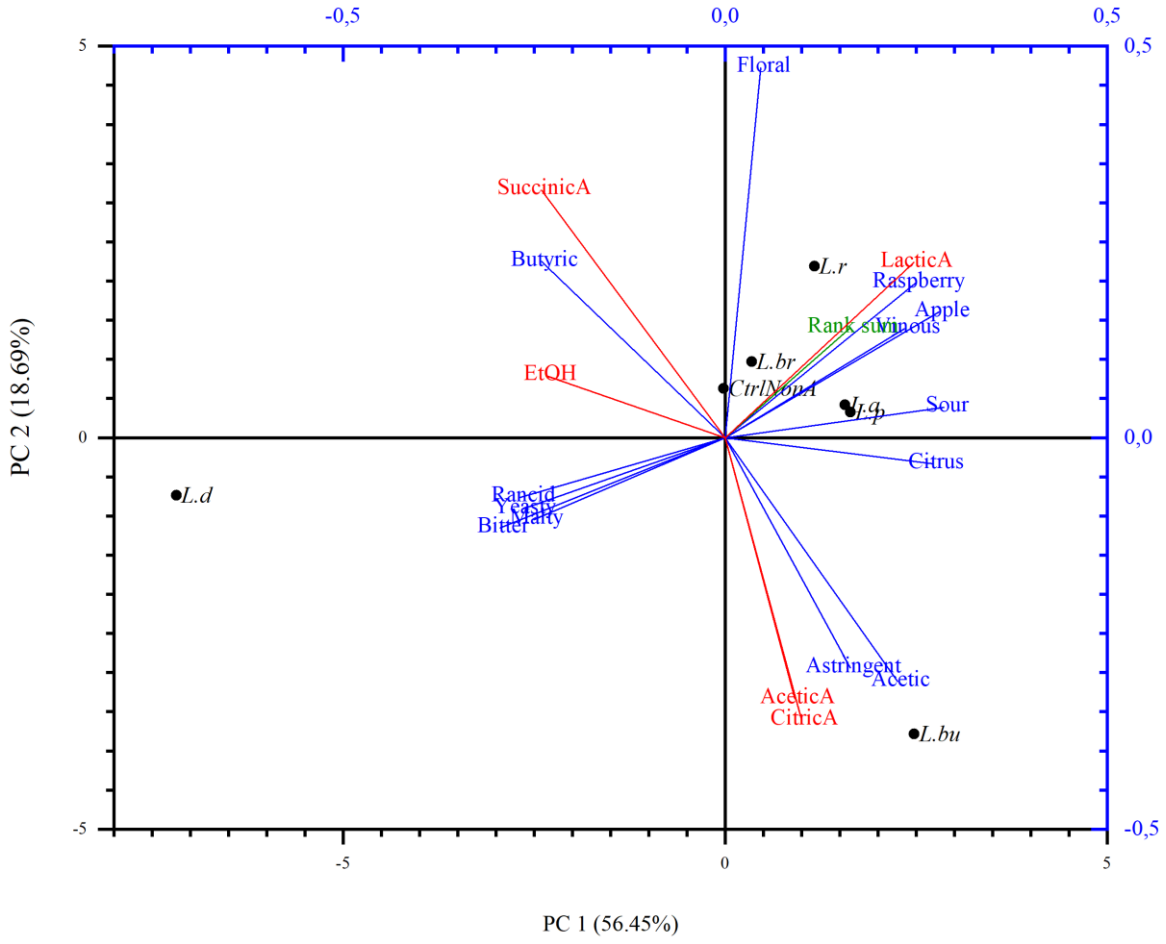

Figure 13. The results of the PCA on a bi-plot. On the blue axes, red vectors are the components of organic acid concentrations and blue vectors the results of the descriptive sensory analysis, with green vector expressing the results of the rank test. On the black axes, black dots signify the corresponding locations of each sample. Generated of the available data with Origin.

Opposing and thus indicating a negative correlation to this combined lactic acid, raspberry, apple, vinous and rank sum -component are the rancid flavour, malty aroma, yeasty flavour and bitter aftertaste attributes. The negative correlation aspect leads to deducing that as the rancid flavour, malty aroma, yeasty flavour and bitter aftertaste are increased, simultaneously rank sum is decreased. To the general direction of the vector and clearly grouped apart

of the other beers is indeed *L. delbrueckii*, which had the lowest score on all positive axis attributes, and the highest on all negative axis attributes.

Another positive correlation group was formed by astringent aftertaste (Astringent, blue), acetic flavour (Acetic, blue), and the concentrations of acetic acid (AceticA, red) and citric acid (CitricA, red). Grouped along these is *L. buchneri*, which indeed had the highest concentration of acetic acid (1.33 ± 0.00 mg ml^{-1}, $p \leq 0.001$) and evaluations of astringent aftertaste of the brewed beers, succeeded only by the commercial control (not pictured in PCA bi-plot due to lack of chemical analysis). Quite clearly by the results of this study, as the acetic acid and citric acid concentrations are increased, so are the evaluations of astringent aftertaste and acetic flavour, but also simultaneously decreased are the lactic and succinic acid concentrations and sour, raspberry and vinous evaluations, as well as the rank sum. Opposing this vector and thus indicating a negative correlation are the succinic acid concentration and evaluation of butyric flavour, resulting in decrease in succinic acid concentration and butyric flavour evaluation as the acetic acid concentration is increased.

Quite notably, the floral aroma is evaluated as most separate of all other sensory attributes and appears as a singular entity. Worth noting is the lack of any floral reference material in the descriptive sensory analysis, leaving each panellist to refer to their respective subjective concepts of floral aroma. Also it is worth noting that only the commercial control sample was the only sample containing dry hops, providing strong floral aroma, as depicted in the radar chart of descriptive analysis (Figure 4). This may have resulted in leaving the evaluation of floral aroma between panellists ambiguous, and thus resulting in a PCA entity appearing separate.

2.3 Discussion

2.3.1 pH, organic acids, and sour taste

Since acidity - arising from the formation of organic acids - is a substantial property of sour beers, measuring pH during fermentation is an eligible method for tracking the state of the LAB fermentation. Prior to inoculation, the wort pH was measured 5.89. For chilled wort, Fix (1998) suggests a pH range of 5.0 – 5.2 and Kunze (1999) 5.3 – 5.6, both of which were exceeded here. pH was not measured during mashing. Most notable perhaps is the overall pH activity in each fermentation: A rapid decrease, followed by a slow but apparent increase. Toh et al. (2018) noted a similar trend in pH activity during a two-week fermentation period

studying beers fermented with *S. cerevisiae* monoculture and a mixed fermentation of *S. cerevisiae* and the yeast *Torulaspora delbrueckii*. In their study the pH levels decreased during the first 48 hours from the initial pH 5.2 to approximately pH 4.5, followed by slow but notable increases in pH values of each beer during the following 12 days of fermentation. According to Kunze (1999) and Bamforth (2001), increase of pH at the end of fermentation suggests yeast autolysis. Many sources (Kunze 1999; Fix 1999; Hornsey 2013) state the deleterious effect of autolysis to the flavour perception of beer. If autolysis occurred here after fermentation, it may have affected the results of the sensory analysis.

Measured lactic acid concentrations during sensory analysis were on average higher than expected (5.40 ± 0.11 mg ml^{-1} in LAB fermented beer). Sources suggest lactic acid concentrations measured in LAB-fermented cereal-based beverages ranging from 0.32 mg ml^{-1} (Pallin et al. 2016) to 5.12 mg ml^{-1} (Coda et al. 2011). In this study, lactic acid concentration ranged from 0.78 mg ml^{-1} (*L. delbrueckii*) to 8.11 mg ml^{-1} (*L. rhamnosus*). It should be stressed, however, that all concentrations measured here may have erroneous increase as a result of possibly overlapping detection of other, unknown components in the chromatograms, interfering with the detection of pure lactic acid. This evaluation is applicable to each chromatogram-based concentration analysis performed in this study.

The taste threshold of acetic acid (0.20 mg ml^{-1} in beer, Siebert 1999) was exceeded most clearly in beer fermented with *L. buchneri* (1.33 ± 0.00 mg ml^{-1}) and was also possibly detected in the descriptive analysis. The concentration of acetic acid exceeded the taste threshold much earlier in the fermentations of this study than in barrel-aged manufactured sour beer. De Roos et al. (2018) detected similar concentrations of acetic acid only after six months of barrel-aging and fermentation by *Acetobacter* species, as opposed to approximately two weeks of tank souring and fermentation with heterofermentative *Lactobacillus* species as demonstrated here. Regardless, this study provides possibly novel insight on prospects of producing sour beers with detectable acetic acid concentrations in vastly shortened fermentation times.

2.3.2 Other flavours

Peyer et al. (2016) reported wort in the range of 10–12 °Bx having maltose concentration of 52–60 mg ml^{-1} , glucose 5–15 mg ml^{-1}, sucrose 1–5 mg ml^{-1}, which correspond with the results measured in this thesis (48, 7.20 and 2.20 mg ml^{-1}, respectively). Since the initial °Bx

in this study was measured to be 13, the actual sugar level may have thus been higher than measured, suggested also by the increase of maltose concentration measured from *L. delbrueckii*-, *L. alimentarius*-, *L. plantarum*-, and *L. buchneri* -fermented beer. The possible increase of maltose in the samples was not explored further, for example by replicating the measurements. Quite surprisingly, while elucidated by the lack of detected sugars in the matured beers, relatively none of the sensory evaluation panellists described any of the beers with "sweet" attributes, indicating a full utilization of the sugars by the fermenting microbes.

Among other components in sour beer as reported by Peyer (2016) was citric acid in concentration of 0.17 mg ml^{-1}, while Coote and Kirsop (1974) reported citric acid in conventional beer in concentrations of approximately 0.1 mg ml^{-1}. Coote and Kirsop (1974) noted no substantial change in citric acid concentration from wort to beer by using four different strains of *S. cerevisiae*, deducing that citric acid levels of beer are determined by the original concentration in wort with no utilization by yeast. This result was later reproduced by Li and Liu (2015), who noted also no change in citric acid concentration from wort to beer from the initial 0.1 mg ml^{-1}, when fermented with *S. cerevisiae*. Notable, however, in this study, is the decrease of citric acid concentration already during the acidification period from the initial concentration of 0.06 mg ml^{-1} (Figure 11) in most beers, except for *L. alimentarius*, for which the significant decrease occurred between 65 and 378 hours. Citric acid was fully depleted in most beers by the sensory evaluation, excluding notably *L. buchneri* and *L. rhamnosus*. Fryer (1970) noted that out of 25 of their studied strains of lactobacilli, 19 were able to utilize 50–100% of the available citrate within 10 days in dairy-based media, most metabolizing possibly to formate. As levels of formic acid were not measured in this study, results comparison is difficult, but gives indication that the citric acid depletion as detected in this study is due to metabolism of lactic acid bacteria, and not the yeast.

Li and Liu (2015) detected levels of approximately 0.1 mg ml^{-1} of succinic acid in beer fermented with *S. cerevisiae* only, while Tyrell (2014) reports levels ranging from 0.05 (beer) to 2.4 mg ml^{-1} (cider). While in this study the detected succinic acid levels remained in most cases at or below the detection threshold of 0.2 mg ml^{-1} in beer (Engan, 1974), in *L. delbrueckii* the detection threshold was exceeded. Taking in consideration the lowest rank sum given to *L. delbrueckii* and the description of succinic acid being reported as having deleterious effect on drinkability of beer (Tyrell 2014), it is possible that the succinic acid concentrations in *L. delbrueckii* did have effect on the results of the sensory evaluation.

2.3.3 Methodological considerations

Other deleterious effects may have been caused by oxygen exposure during fermentation. Staling of beer may have occurred, which may have further affected the results of the sensory evaluation. By replacing as much ambient air with CO_2 during bottling as possible, some of the unwanted effects may have been avoided. Also, as described by Bamforth (2001), the exposure to O_2 during yeast fermentation promotes to pH decrease rate, as the yeast proton excretion is stimulated. This might in part explain the notable decrease of pH in the non-acidified control beer, however, more likely seems to be the case of a contamination by unintentionally introduced microbe. For conventional, nonacidified beers in general, more moderate decrease in pH is usually observed, reaching according to Kunze (1999) a typical range of 4.3 – 4.6. For beer fermented with *S. cerevisiae* Safale US-05 strain, Liu and Quek (2016) reported a terminal pH of approximately 4.0. Further supporting the hypothesis of contamination in the nonacidified control beer and *L. delbrueckii* are the notably detected concentrations of lactic acid (3.48 ± 0.08 mg ml^{-1}) and acetic acid (1.33 ± 0.14 mg ml^{-1}), both of which exceed general levels described in literature.

In addition to these possible contaminations, other possible sources of error in the performance of the experimental research were in the lack of completely non-inoculated control wort, lack of plating of the control beer during fermentation, low number of sensory analysis panellists and singular measurements of sugar concentrations.

3 CONCLUSIONS

The objectives of the thesis were met while answering to the research questions. Sour beers manufactured with different LAB species differed in sensory characteristics, organic acids and ethanol. Increased lactic acid concentration was related to increased rank as evaluated by overall preference, showing positive grouped correlation with sensory evaluation of raspberry aroma, apple flavour and vinous flavour. The group appeared to have negative correlation with another group comprising of rancid flavour, malty aroma, yeasty flavour and bitter aftertaste attributes.

Acetic acid was measured in sour beers manufactured with the use of obligatory heterofermentative LAB species *L. buchneri* and *L. brevis*. In both beers, the detection threshold of acetic acid in beer was exceeded, while *L. buchneri* was evaluated highest with acetic flavour

attribute. In both beers and confirming the heterofermentative nature of bacteria, minute levels of ethanol was also detected. Encouraging results of usage of these bacteria in producing sour beers with sometimes desired acetic flavours were gained.

Novel LAB in the use of sour beer manufacture, *L. alimentarius,* was reported here to the best of knowledge for the first time, with encouraging results. According to the results of this thesis, *L. alimentarius* provides fast acidification of the wort media with highly increased cell growth, as well as flavour profiles similar with beers manufactured with more commonly used *L. plantarum*.

The results of this thesis may provide useful insight and information for breweries planning or initiating a sour beer product development project. However, as wort media composition, buffering capacity of the media or changes in the fermentation conditions, such as fermentation temperature or O_2 availability were not controlled, the results of this thesis can function merely as starting points for more rigorous and robust experiments.

Sour beers present a possibility to gain new consumers to drinking beer. With flavour profiles conventionally not associated with beer, characterized here with attributes such as apple, raspberry, citrus and vinous, sour beers may attract novel consumer base from the wine- and cider-drinking consumers. For the general lager beer -drinking consumers, complex and surprising sour beers may very well give new perspectives around the assumedly simple concept of beer.

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APPENDICES

Appendix 1. ASBC Reference handouts given to the sensory evaluation panellists in training sessions of descriptive analysis. From ASBC Methods of analysis: Sensory analysis method 12 - Flavor terminology and reference standards.

TABLE I
Recommended Descriptors with Particular Relevance for Odor (O), Taste (T), Mouthfeel (M), Warming (W), and After flavor (Af)

Class Term	First Tier	Second Tier	Relevance	Comments, Synonyms, Definitions	Reference Standard
Class 1—Aromatic, Fragrant, Fruity, Floral					
	0110 Alcoholic		OTW	General effect of ethanol and higher alcohols	Ethanol, 50 g/L
		0111 Spicy	OTW	Allspice, nutmeg, peppery, eugenol; see also 1003 Vanilla	Eugenol, 120 µg/L
		0112 Vinous	OTW	Bouquet, fusely, wine-like	(White wine)
	0120 Solvent-like		OT	Like chemical solvents	
		0121Plastics	OT	Plasticizers	
		0122 Can-liner	OT	Lacquer-like	
		0123 Acetone	OT		(Acetone)
	0130 Estery		OT	Like aliphatic esters	
		0131 Isoamyl acetate	OT	Banana, peardrop	(Isoamyl acetate)
		0132 Ethyl hexanoate	OT	Apple-like with note of aniseed; see also 0142 Apple	(Ethyl hexanoate)
		0133 Ethyl acetate	OT	Light fruity, solvent-like; see also 0120 Solvent-like	(Ethyl acetate)
	0140 Fruity		OT	Of specific fruits or mixtures of fruits	
		0141 Citrus	OT	Citral, grapefruit, lemony, orange rind	
		0142 Apple	OT		
		0143 Banana	OT		
		0144 Black currant	OT	Black currant fruit; for black currant leaves use 0819 Catty	
		0145 Melony	OT		(6-Nonenal, *cis*- or *trans*-)
		0146 Pear	OT		
		0147 Raspberry	OT		
		0148 Strawberry	OT		
	0150 Acetaldehyde		OT	Green apples, raw apple skin, bruised apples	(Acetaldehyde)
	0160 Floral		OT	Like flowers, fragrant	
		0161 2-Phenylethanol	OT	Rose-like	(2-Phenylethanol)
		0162 Geraniol	OT	Rose-like, different from 0161; taster should compare pure chemicals	(Geraniol)
		0163 Perfumy	OT	Scented	(Exaltolide musk)
	0170 Hoppy		OT	Fresh hop aroma; use with other terms to describe stale hop aroma; does not include hop bitterness (see 1200 Bitter)	
		0171 Kettle-hop	OT	Flavor imparted by aroma hops boiled in kettle	
		0172 Dry-hop	OT	Flavor imparted by dry hops added in tank or cask	
		0173 Hop oil	OT	Flavor imparted by addition of distilled hop oil	
Class 2—Resinous, Nutty, Green, Grassy					
	0210 Resinous		OT	Fresh sawdust, resin, cedarwood, pinewood, sprucy, terpenoid	
		0211 Woody	OT	Seasoned wood (uncut)	
	0220 Nutty		OT	As in brazil nut, hazelnut, sherry-like	
		0221 Walnut	OT	Fresh (not rancid) walnut	
		0222 Coconut	OT		
		0223 Beany	OT	Bean soup	(2,4,7-Decatrienal)
		0224 Almond	OT	Marzipan	(Benzaldehyde)
	0230 Grassy		OT		
		0231 Freshly cut grass	OT	Green, crushed green leaves, leafy, alfalfa	(*cis*-3-Hexenol)
		0232 Straw-like	OT	Hay-like	
Class 3—Cereal					
	0310 Grainy		OT	Raw grain flavor	
		0311 Husky	OT	Husk-like, chaff, *Glattwasser*	
		0312 Corn grits	OT	Maize grits, adjuncty	
		0313 Mealy	OT	Like flour	
	0320 Malty		OT		
	0330 Worty		OT	Fresh wort aroma; use with other terms to describe infected wort (e.g., 0731 Parsnip/celery)	
Class 4—Caramelized, Roasted					
	0410 Caramel		OT	Burnt sugar, toffee-like	
		0411 Molasses	OT	Black treacle, treacly	
		0412 Licorice	OT		
	0420 Burnt		OTM	Scorched aroma, dry mouthfeel, sharp, acrid taste	
		0421 Bread crust	OTM	Charred toast	
		0422 Roast barley	OTM	Chocolate malt	
		0423 Smoky	OT		

TABLE I (continued)

Class Term	First Tier	Second Tier	Relevance	Comments, Synonyms, Definitions	Reference Standard
Class 5—Phenolic					
0500 Phenolic			OT		
		0501 Tarry	OT	Pitch, faulty pitching of containers	
		0502 Bakelite	OT		
		0503 Carbolic	OT	Phenol, C_6H_5OH	
		0504 Chlorophenol	OT	Trichlorophenol (TCP), hospital-like	
		0505 Iodoform	OT	Iodophors, hospital-like, pharmaceutical	
Class 6—Soapy, Fatty, Diacetyl, Oily, Rancid					
	0610 Fatty acid		OT		
		0611 Caprylic	OT	Soapy, fatty, goaty, tallowy	(Octanoic acid)
		0612 Cheesy	OT	Dry, stale cheese, old hops } Hydrolytic rancidity	(Isovaleric acid)
		0613 Isovaleric	OT		
		0614 Butyric	OT	Rancid butter	Butyric acid, 3 mg/L
	0620 Diaccetyl		OT	Butterscotch, buttermilk	Diacetyl, 0.2–0.4 mg/L
	0630 Rancid		OT	Oxidative rancidity	
		0631 Rancid oil	OTM		
	0640 Oily		OTM		
		0641 Vegetable oil	OTM	As in refined vegetable oil	
		0642 Mineral oil	OTM	Gasoline (petrol), kerosene (paraffin), machine oil	
Class 7—Sulfury					
0700 Sulfury			OT		
	0710 Sulfitic		OT	Sulfur dioxide, striking match, choking, sulfurous-SO_2	(KMS)
	0720 Sulfidic		OT	Rotten egg, sulfury-reduced, sulfurous-RSH	
		0721 H_2S	OT	Rotten egg	(H_2S)
		0722 Mercaptan	OT	Lower mercaptans, drains, stench	(Ethyl mercaptan)
		0723 Garlic	OT		
		0724 Lightstruck	OT	Skunky, sunstruck	
		0725 Autolysed	OT	Rotting yeast; see also 0740 Yeasty	
		0726 Burnt rubber	OT	Higher mercaptans	
		0727 Shrimp-like	OT	Water in which shrimp have been cooked	
	0730 Cooked vegetable		OT	Mainly dialkyl sulfides, sulfurous-RSR`	
		0731 Parnsip/Celery	OT	Effect of wort infection	
		0732 DMS	OT	(Dimethyl sulfide)	DMS, 100 µg/L
		0733 Cooked Cabbage	OT	Overcooked green vegetables	
		0734 Cooked sweet corn	OT	Cooked maize, canned sweet corn	
		0735 Cooked tomato	OT	Tomato juice (processed), tomato ketchup	
		0736 Cooked onion	OT		
	0740 Yeasty		OT	Fresh yeast, flavor of heated thiamine; see also 0725 Autolysed	
		0741 Meaty	OT	Brothy, cooked meat, meat extract, peptone, yeast broth	
Class 8—Oxidixed, Stale, Musty					
0800 Stale			OTM	Old beer, overaged, overpasteurized	(Heat with air)
	0810 Catty		OT	Black currant leaves, ribes, tomato plants, oxidized beer	(*p*-Menthane-8- thiol-3-one)
	0820 Papery		OT	Initial stage of staling, bready (stale bread crumb), cardboard, old beer, oxidized	(5-Methylfurfural, 25 mg/L)
	0830 Leathery		OTM	Later stage of staling, often used in conjunction with 0211 Woody	
	0840 Moldy		OT	Cellar-like, leaf mold, woodsy	
		0841 Earthy	OT	Actinomycetes, damp soil, freshly dug soil, diatomaceous earth	(Geosmin)
		0842 Musty	OT	Fusty	
Class 9—Sour Acidic					
0900 Acidic			OT	Pungent aroma, sharpness of taste, mineral acid	
	0910 Acetic		OT	Vinegar	(Acetic acid)
	0920 Sour		OT	Lactic, sour milk; use with 0141 Citrus for citrus-sour	
Class 10—Sweet					
1000 Sweet			OT		Sucrose, 7.5 g/L
		1001 Honey	OT	Can occur as effect of beer staling (e.g., odor of stale beer in glass), oxidized (stale) honey	
		1002 Jam-like	OT	May be qualified by subclasses of 0140 Fruity	
		1003 Vanilla	OT	Custard powder, vanillin	(Vanillin)

TABLE I (continued)

Class Term	First Tier	Second Tier	Relevance	Comments, Synonyms, Definitions	Reference Standard
		1004 Primings	OT		
		1005 Syrupy	OTM	Clear (golden) syrup	
		1006 Oversweet	OT	Sickly sweet, cloying	
Class 11—Salty					
1100 Salty			T		Sodium chloride, 1.8 g/L
Class 12—Bitter					
1200 Bitter			TAf		(Isohumulone)
Class 13—Mouthfeel					
	1310 Alkaline		TMAf	Flavor imparted by accidental admixture of alkaline detergent	(Sodium bicarbonate)
	1320 Mouthcoating		MAf	Creamy, *onctueux* (Fr.)	
	1330 Metallic		OTMAf	Iron, rusty water, coins, tinny, inky	(Ferrous ammonium sulfate)
	1340 Astringent		MAf	Mouth puckering, puckery, tannin-like, tart	Quercitrin, 240 mg/L[a]
		1341 Drying	MAf	Unsweet	
	1350 Powdery		OTM	O—Dusty cushion, irritating, (with 0310 Grainy) mill- room smell TM—Chalky, particulate, scratchy, silicate-like, siliceous	
	1360 Carbonation		M	CO_2 content	
		1361 Flat	M	Undercarbonated	60% of normal CO_2 content for the product
		1362 Gassy	M	Overcarbonated	140% of normal CO_2 content for the product
	1370 Warming		WMAf	See 0110 Alcoholic and 0111 Spicy	
Class 14—Fullness					
	1410 Body		OTM	Fullness of flavor and mouthfeel	
		1411 Watery	TM	Thin, seemingly diluted	
		1412 Characterless	OTM	Bland, empty, flavorless	
		1413 Satiating	OTM	Extra full, filling	
		1414 Thick	TM	Viscous, *epais* (Fr.)	

[a] Quercitrin is both astringent and bitter.

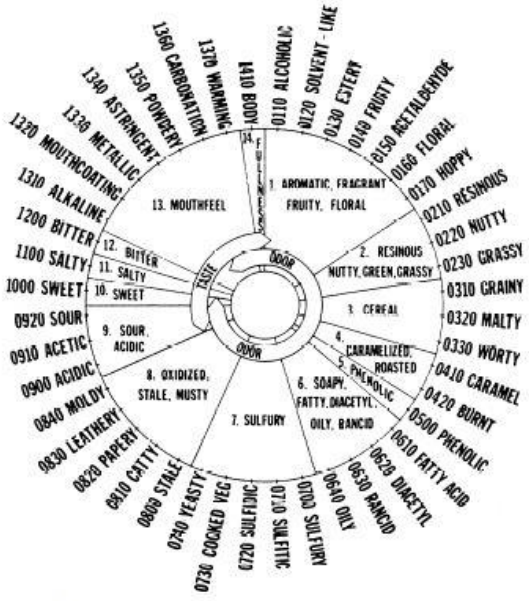


Fig. 1. Flavor Wheel, showing class terms and first-tier terms.

Flavor Reference Standards

The reference standards were tested and selected in 1975–1982 by members of the working group (Refs. 1,3) (Table II). Sniff bottles, despite their convenience and reusability, gave poorer recognition, more errors, and less precise flavor descriptions and were rejected in favor of a system of standards dissolved in beer. Users are referred to Reference 3 for details of purification, preparation of stock solutions, stability, addition to beer, and demonstration to tasters.

An ideal reference compound is sodium chloride, which represents the term 1100 SALTY perfectly. It is easily purified and can even be used without purification. It is stable, nontoxic, and nonreactive in beer. It does not affect foam, color, or clarity, and is soluble at 9× and even at 81× threshold concentration. Its difference threshold when added to beer is well defined, and it does not show a highly skewed or bimodal distribution of individual thresholds. A second near-ideal reference compound is sucrose, for the term 1000 SWEET. All other substances listed cause one or more problems that make them less than ideal reference substances.

Appendix 2. User interface screens of ranking test, as viewed by the user in Fizz.

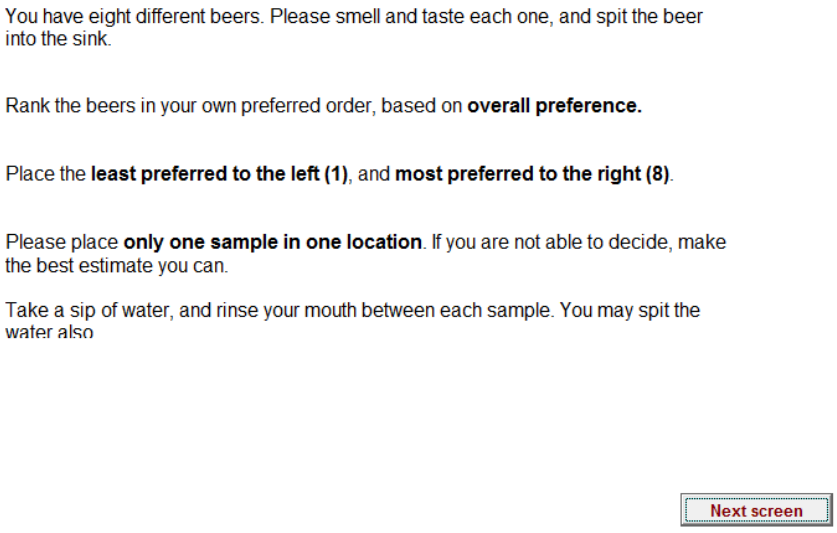


Figure A2.1. Information screen for the rank test.

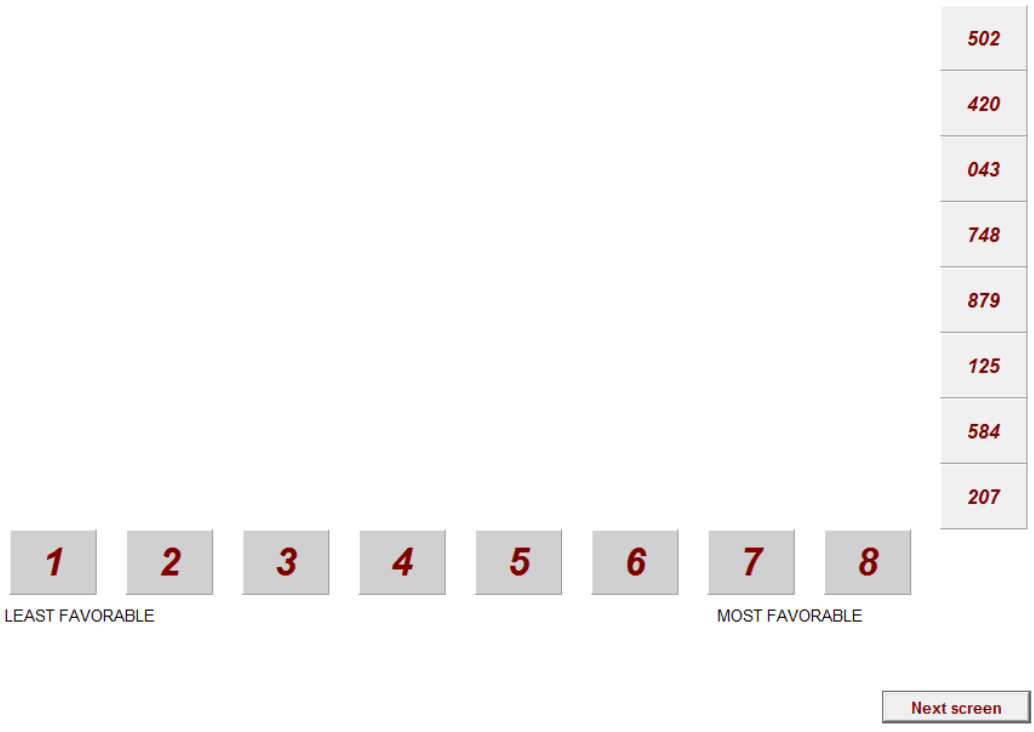


Figure A2.2. Rank test screen. The user drags and drops sample codes from the column on the right to the Likert scale below.

Appendix 3. User interface screens of descriptive analysis, as viewed by the user in Fizz.

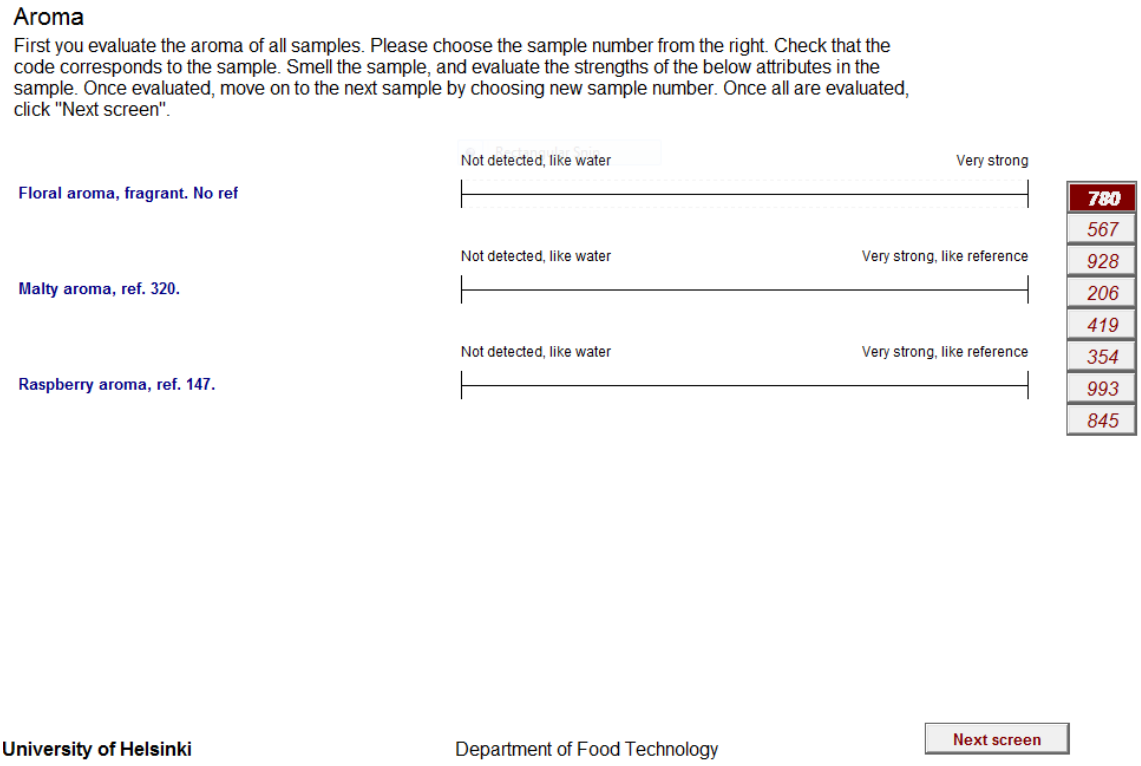


Figure A3.1. User interface screen for evaluating the intensities of aroma attributes.

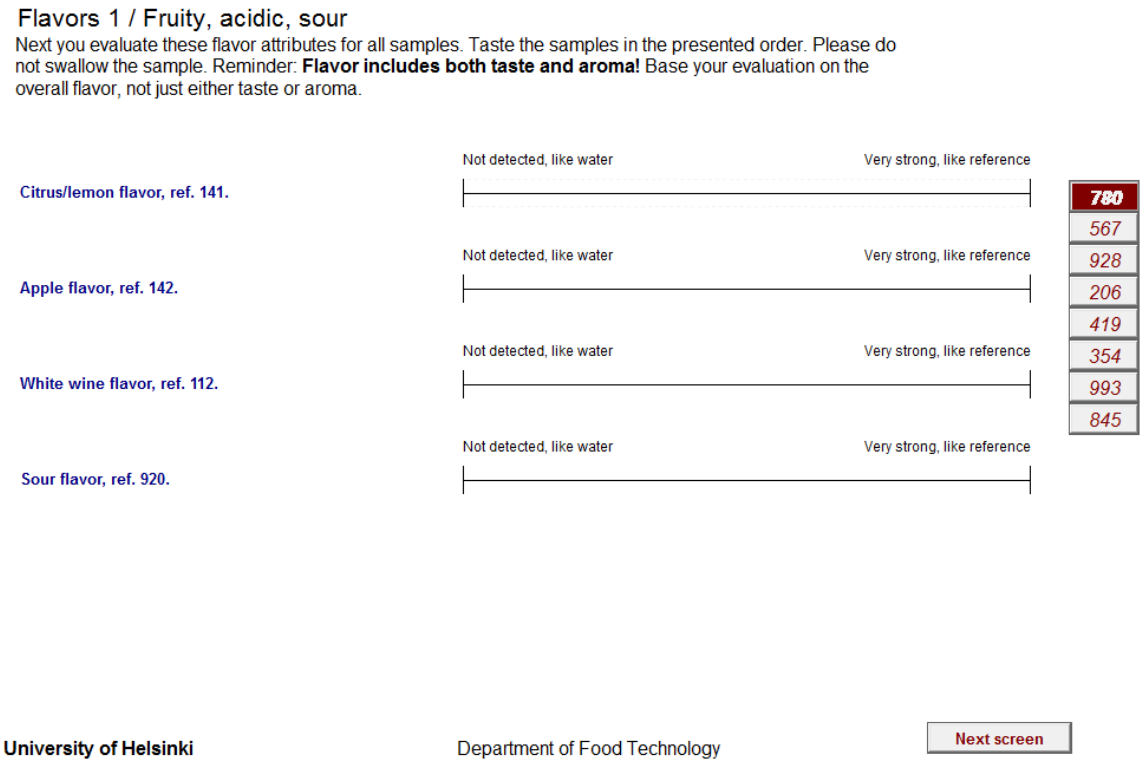


Figure A3.2. User interface screen for evaluating the intensities of fruity, acidic and sour flavour attributes.

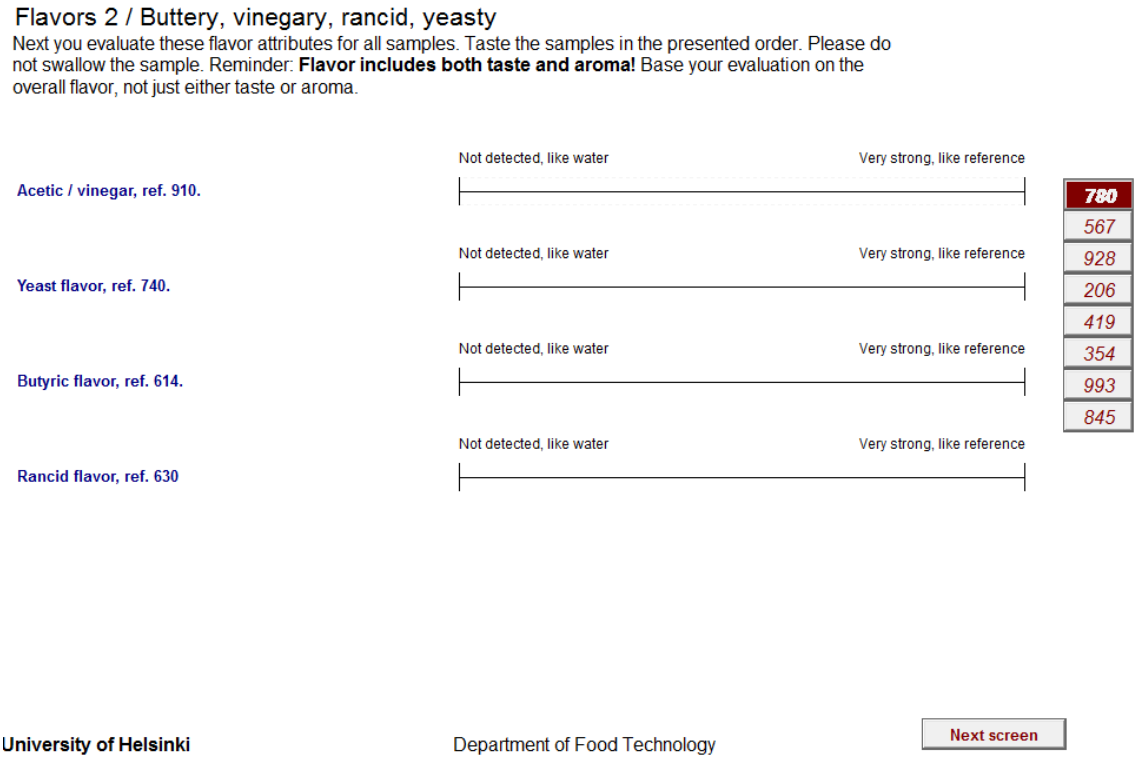


Figure A3.3. User interface screen for evaluating the intensities of buttery, vinegary, rancid and yeasty flavour attributes.

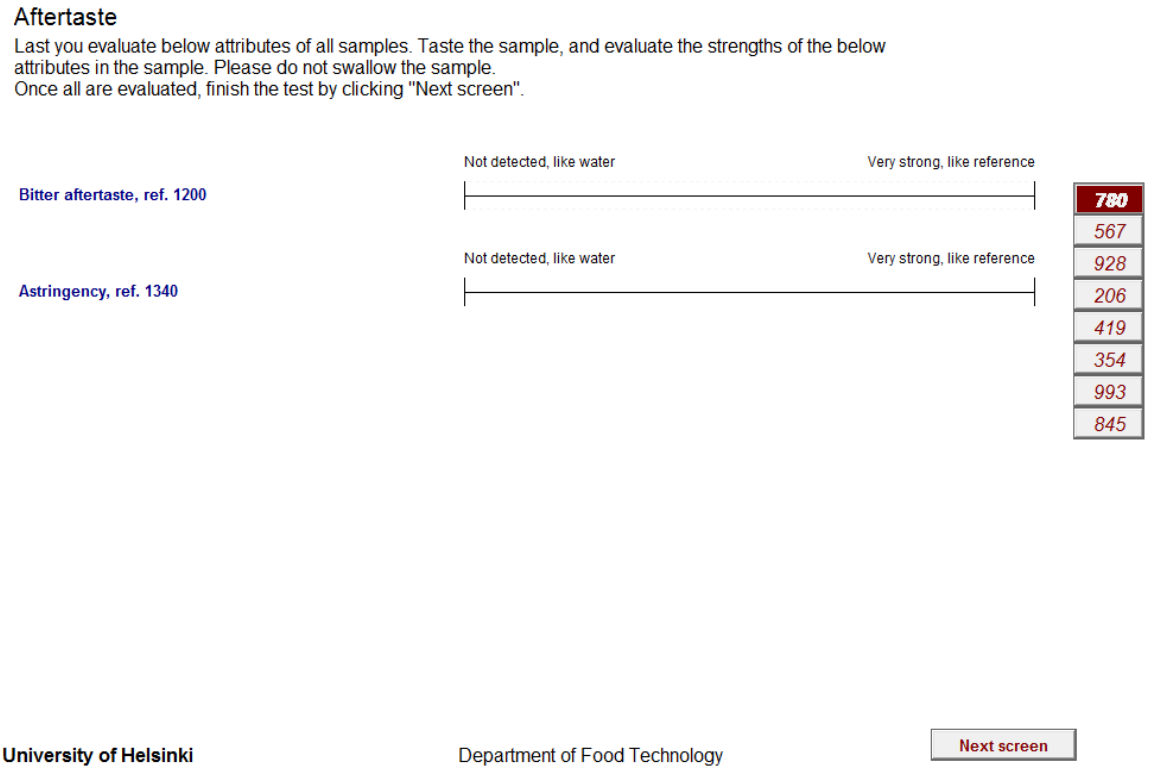
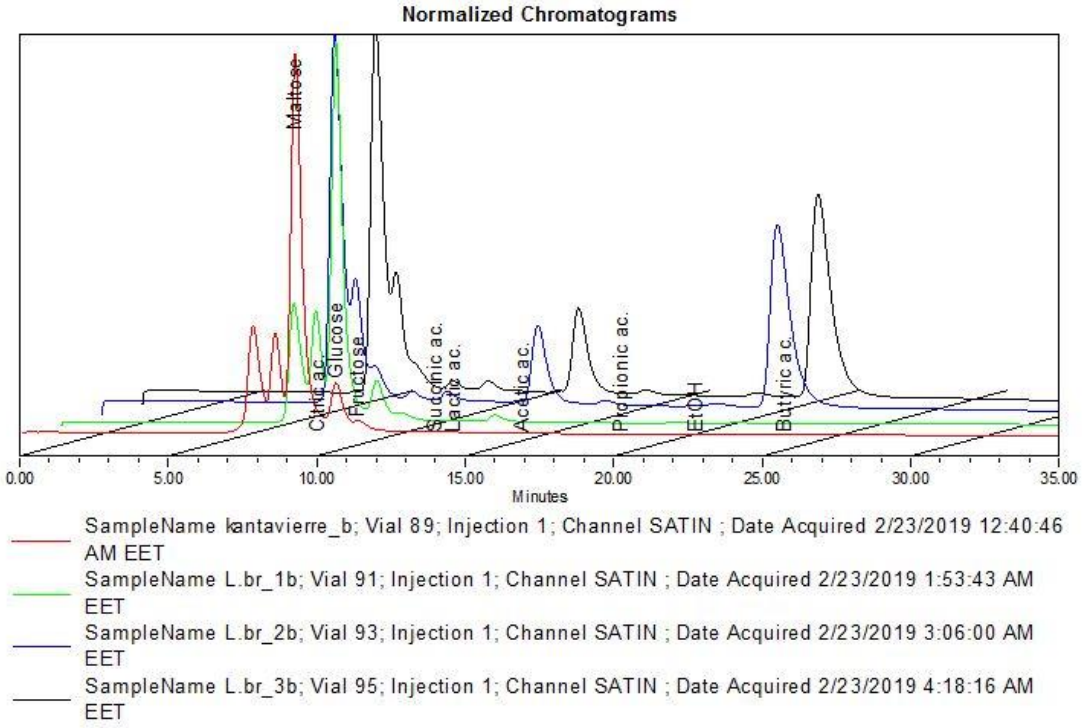


Figure A3.4. User interface screen for evaluating the intensities of buttery, vinegary, rancid and yeasty flavour attributes.

Appendix 4. The eluent gradient used in HPAEC-PAD.

Time (min)	200 mM NaOH	Milli-Q water
0	1 %	99 %
4	1 %	99 %
30	30 %	70 %
38	100 %	0 %
48	100 %	0 %
50	1 %	99 %
60	1 %	99 %

Appendix 5. An example chromatogram from PDA/RI results for *L. brevis*.



Component Summary For Concentration

	Sample Name	Inj	Vial	Injection Volume (uL)	Dilution	Processing Method	Date Processed
1	kantavierre_b	1	89	40.00	1.00000	Santeri_RI_40C_250219	2/25/2019 2:57:28 PM EET
2	L.br_1b	1	91	40.00	1.00000	Santeri_RI_40C_250219	2/25/2019 3:00:17 PM EET
3	L.br_2b	1	93	40.00	1.00000	Santeri_RI_40C_250219	2/25/2019 3:03:37 PM EET
4	L.br_3b	1	95	40.00	1.00000	Santeri_RI_40C_250219	2/25/2019 3:20:36 PM EET

Component Summary For Concentration

	Maltose	Citric ac.	Glucose	Fructose	Succinic ac.	Lactic ac.	Acetic ac.	Propionic ac.	EtOH
1	17.538		2.061	0.428					
2	17.705		1.827	0.407		0.465			0.112
3	0.468		0.054	0.084		1.854	0.110		9.700
4	0.413		0.117	0.117	0.037	2.078	0.105	0.085	10.761

Appendix 6. Results of the descriptive sensory analysis. N=16 for each data point. A=Aroma attribute, F=Flavour attribute, AT=Aftertaste attribute.

Attribute	Sample							
	L. a	*L. br*	*L. bu*	*L. d*	*L. p*	*L. r*	Ctrl Comm	Ctrl NonA
Floral (A) (***	3.13 ± 3.11^{b}	2.81 ± 2.94^{b}	1.77 ± 1.87^{b}	2.35 ± 2.73^{b}	2.75 ± 2.36^{b}	3.44 ± 3.54^{b}	7.13 ± 2.75^{a}	3.51 ± 3.24^{b}
Malty (A) (**	2.19 ± 2.23^{ab}	1.93 ± 2.05^{a}	2.88 ± 2.28^{ab}	4.73 ± 3.04^{b}	2.86 ± 2.33^{ab}	3.11 ± 2.95^{ab}	1.15 ± 1.93^{a}	3.01 ± 2.29^{ab}
Raspberry (A) (*	3.43 ± 2.76^{ab}	3.60 ± 2.58^{ab}	3.68 ± 2.94^{ab}	1.34 ± 1.21^{a}	3.52 ± 3.19^{ab}	5.00 ± 3.01^{b}	4.63 ± 3.67^{b}	4.07 ± 2.88^{ab}
Citrus (F) (***	4.55 ± 3.04^{b}	4.38 ± 3.11^{b}	4.38 ± 2.10^{b}	1.04 ± 2.26^{a}	4.75 ± 2.58^{b}	3.17 ± 3.20^{ab}	5.08 ± 3.24^{b}	2.83 ± 2.87^{ab}
Apple (F)	3.45 ± 2.96	3.46 ± 2.60	3.43 ± 2.70	1.53 ± 1.79	3.35 ± 2.25	3.96 ± 3.03	3.98 ± 3.35	3.61 ± 2.41
Vinous (F) (**	2.73 ± 2.61^{ab}	3.01 ± 2.85^{ab}	3.48 ± 3.02^{ab}	1.02 ± 1.62^{a}	2.73 ± 3.11^{ab}	4.55 ± 3.40^{b}	5.26 ± 2.90^{b}	3.58 ± 2.48^{ab}
Sour (F) (**	5.80 ± 2.58^{b}	5.67 ± 2.79^{b}	5.38 ± 3.32^{b}	2.01 ± 2.64^{a}	5.81 ± 2.82^{b}	4.71 ± 3.09^{ab}	5.21 ± 2.63^{b}	4.57 ± 2.91^{ab}
Acetic (F)	2.96 ± 2.18	2.34 ± 2.27	3.61 ± 2.15	1.86 ± 1.98	3.17 ± 2.17	2.28 ± 2.41	2.40 ± 2.01	2.36 ± 2.54
Yeasty (F) (***	1.88 ± 1.95^{ab}	3.14 ± 2.51^{bc}	2.00 ± 2.29^{abc}	4.27 ± 3.15^{c}	1.55 ± 1.73^{ab}	1.46 ± 1.93^{ab}	0.36 ± 0.28^{a}	1.95 ± 1.77^{ab}
Butyric (F)	1.94 ± 1.81	1.98 ± 2.06	1.41 ± 1.69	3.15 ± 2.75	2.33 ± 2.07	2.57 ± 2.57	1.06 ± 1.70	1.95 ± 1.91
Rancid (F)	2.21 ± 1.93	2.62 ± 2.50	2.36 ± 1.61	3.70 ± 2.65	1.65 ± 1.94	2.21 ± 2.38	1.78 ± 2.12	2.86 ± 1.97
Bitter (AT)	2.48 ± 2.40	2.81 ± 3.00	2.93 ± 2.84	5.48 ± 2.30	2.69 ± 3.00	2.66 ± 2.72	3.44 ± 3.40	3.40 ± 3.30
Astringent (AT)	3.00 ± 2.55	2.52 ± 2.72	3.55 ± 2.84	2.63 ± 2.89	3.33 ± 2.65	2.78 ± 3.19	3.59 ± 2.99	3.31 ± 3.53

L. a = L. alimentarius; L. br = L. brevis; L. bu = L. buchneri;
L. d = L. delbrueckii; L. p = L. plantarum; L. r = L. rhamnosus.
*) The attribute contains mean differences at $p \leq 0.05$ significance level.
**) The attribute contains mean differences at $p \leq 0.01$ significance level.
***) The attribute contains mean differences at $p \leq 0.001$ significance level.
a – c) Statistically significant homogenous subsets.

Appendix 7. Organic acid and ethyl alcohol concentrations in the 960 h samples in mg ml^{-1}. N=2 samples for each measurement.

Component	Concentration in sample (mg ml^{-1})						
	L.r	*L.bu*	*L.a*	*L.p*	*L.br*	*L.d*	Ctrl
Lactic acid (***	8.10 ± 1.60^{d}	4.69 ± 0.06^{bc}	6.76 ± 0.27^{cd}	6.77 ± 0.24^{cd}	5.24 ± 0.02^{bc}	0.78 ± 0.00^{a}	3.48 ± 0.07^{b}
Acetic acid (***	0.00 ± 0.00^{e}	1.33 ± 0.00^{h}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.29 ± 0.03^{f}	0.00 ± 0.00^{e}	1.08 ± 0.13^{g}
Succinic acid (**	0.21 ± 0.01^{jk}	0.06 ± 0.03^{i}	0.14 ± 0.00^{ij}	0.17 ± 0.00^{k}	0.20 ± 0.02^{jk}	0.28 ± 0.05^{k}	0.20 ± 0.00^{jk}
Citric acid (*	0.01 ± 0.01^{lm}	0.01 ± 0.00^{m}	0.00 ± 0.00^{l}	0.00 ± 0.00^{l}	0.00 ± 0.00^{l}	0.00 ± 0.00^{l}	0.00 ± 0.00^{l}
EtOH (***	54.58 ± 0.18^{n}	52.55 ± 0.57^{n}	51.83 ± 1.29^{n}	54.08 ± 0.87^{n}	54.2 ± 0.58^{n}	59.67 ± 1.02^{o}	59.42 ± 1.02^{o}

L. a = L. alimentarius; L. br = L. brevis; L. bu = L. buchneri;
L. d = L. delbrueckii; L. p = L. plantarum; L. r = L. rhamnosus.
$^{a-o)}$ Statistically homogenous subsets for each row, respectively.
$^{*)}$ The mean differences are significant at $p \leq 0.05$
$^{**)}$ The mean differences are significant at $p \leq 0.01$.
$^{***)}$ The mean differences are significant at $p \leq 0.001$.

Appendix 8. Extracted coefficient eigenvector values for PC1, PC2, PC3 and PC4, explaining 93.57% of variation within the data.

Attribute	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3	Coefficients of PC4
Malty	-0.25061	-0.10464	0.12747	0.38156
Floral	0.04665	0.47249	0.192	-0.04707
Astringent	0.16379	-0.29432	0.21783	0.01448
Raspberry	0.24901	0.19724	0.22893	0.21468
Citrus	0.27232	-0.03355	-0.23781	-0.22047
Apple	0.27974	0.16036	0.15101	0.04023
Vinous	0.23458	0.13922	0.28665	0.28823
Sour	0.28657	0.03875	-0.14118	-0.23232
Acetic	0.2258	-0.31159	-0.13858	0.00327
Yeasty	-0.26608	-0.09128	-0.10603	-0.23369
Butyric	-0.24051	0.22473	-0.10433	0.30628
Rancid	-0.26808	-0.0763	0.18113	-0.11118
Bitter	-0.29458	-0.11543	0.08783	0.0281
Rank sum	0.16357	0.14005	0.42985	-0.29838
LacticA	0.24698	0.22356	-0.18513	0.27077
AceticA	0.09129	-0.3336	0.44582	-0.1734
EtOH	-0.23318	0.07891	0.3901	-0.10678
CitricA	0.10022	-0.36093	0.11326	0.50318
SuccinicA	-0.24051	0.31639	0.03001	-0.00577
Percentage of variance	56.45 %	18.69 %	12.06 %	6.38 %